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(54) Title: MULTIPOTENT NEURAL STEM CELLS FROM PERIPHERAL TISSUES AND USES THEREOF

(57) Abstract: This invention relates to multipotent neural stem cells, purified from the peripheral nervous system of mammals, capable of differentiating into neural and non-neural cell types. These stem cells provide an accessible source for autologous trans-
plantation into CNS, PNS, and other damaged tissues.



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MULTIPOTENT NEURAL STEM CELLS FROM
PERIPHERAL TISSUES AND USES THEREOF

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Cross Reference To Related Applications

This application is a continuation-in-part of and claims priority from U.S. Application Serial No. 09/670,049, filed September 25, 2000, which application is a continuation-in-part of application Serial No. 09/490,422, filed January 24, 2000, each of which is hereby incorporated by reference.

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Background of the Invention

The present invention relates to multipotent stem cells (MSCs), or multipotent neural stem cells (MNSCs), purified from peripheral tissues including peripheral tissues containing sensory receptors such as the skin, olfactory
15 epithelium, and tongue.

There are a number of diseases of the central nervous system ("CNS") which have a devastating effect on patients. These diseases are debilitating, often incurable, and include, for example, Alzheimer's disease, Huntington's disease, Parkinson's disease, and Multiple Sclerosis.

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By way of example, Parkinson's disease is a progressive degenerative disorder of unknown cause. In healthy brain tissue, dopaminergic neurons extend from the substantia nigra of the brain into the neighboring striatum. In Parkinson's disease, these dopaminergic neurons die.

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There are a number of methods to treat Parkinson's disease. One method is to treat humans having Parkinson's disease with L-DOPA. A second method is to transplant cells into the substantia nigra or striatum. Transplanted cells replace endogenous cells that are lost as a consequence of disease progression. An animal model of Parkinson's disease is an MPTP-treated non-human primate. The MPTP-

treated animals have been transplanted with dopamine-rich embryonic neurons with some success.

To date, the cells used for neural transplant have been collected from the developing brains of aborted fetuses. Aside from the ethical considerations, the method from a practical standpoint is unlikely to provide a sufficient amount of neural tissue to meet the demands. Thus, another source of cells for transplantation is desirable.

Stem cells are undifferentiated cells that exist in many tissues of embryos and adult mammals. In embryos, blastocyst stem cells are the source of cells which differentiate to form the specialized tissues and organs of the developing fetus. In adults, specialized stem cells in individual tissues are the source of new cells, replacing cells lost through cell death due to natural attrition, disease, or injury. Stem cells may be used as substrates for producing healthy tissue where a disease, disorder, or abnormal physical state has destroyed or damaged normal tissue.

MSCs, or MNSCs, may be used as a source of cells for transplantation. The stem cells may themselves be transplanted or, alternatively, they may be induced to produce differentiated cells (*e.g.*, neurons, oligodendrocytes, Schwann cells, or astrocytes) for transplantation. Transplanted stem cells may also be used as vectors for the expression of therapeutic molecules, such as growth factors, cytokines, anti-apoptotic proteins, and the like. Thus, stem cells are a potential source of cells for alternative treatments of diseases involving loss of cells or tissues.

The safest type of tissue graft (using stem cells or otherwise) is one that comes from self (an autologous tissue source). Autologous tissue sources are widely used in procedures such as bone transplants and skin transplants because a source of healthy tissue is readily accessible for transplant to a damaged tissue site. In brain diseases, such as Parkinson's disease, healthy dopaminergic neuronal brain tissue may exist at other sites in the brain, but attempts to transplant these neurons may harm the site where the healthy neurons originate. Multipotent stem cells that can be differentiated into dopaminergic neurons may be available at other sites from which

they may be transplanted, but the CNS, particularly the brain, is physically difficult to access.

In several tissues, stem cells have been purified and characterized. For example, neural stem cells have been purified from the mammalian forebrain
5 (Reynolds and Weiss, Science 255:1707-1710, 1992) and these cells were shown to be capable of differentiating into neurons, astrocytes, and oligodendrocytes. PCT publications WO 93/01275, WO 94/16718, WO 94/10292 and WO 94/09119 describe uses for these cells. It could be impractical or impossible, however, to first access brain or other CNS tissue for biopsy and then again for transplant in patients
10 with weakened health. It would be very useful if there were accessible stem cells capable of differentiating into CNS cell types, such as dopaminergic neurons; such cells would be a source of cells for autologous transplants.

Thus, there is a clear need to develop methods for identifying from accessible tissues multipotent stem cells that can act as a source of cells that are
15 transplantable to the CNS, PNS, or other tissues *in vivo* in order to replace damaged or diseased tissue.

Summary of the Invention

We have substantially purified multipotent stem cells, or MNSCs, from
20 the peripheral tissue of postnatal mammals, including juvenile and adult mammals. We have identified skin as a source of MNSCs and provide methods for the purification of skin-derived MNSCs, thus simplifying the harvesting of cells for transplantation relative to previous methods. The MNSCs possess desirable features in that they are multipotent and self-renewing. The cells can be repeatedly passaged
25 and differentiated into numerous cell types of the CNS, including astrocytes, oligodendrocytes, and neurons. The MSCs of this invention express nestin, an immunological marker of stem cells and progenitor cells, as well as fibronectin. The cells are capable of differentiating as dopaminergic neurons, and thus are useful source of dopaminergic neurons for homotypic grafts into Parkinson's Disease

patients. The MNSCs have been demonstrated to make smooth muscle cells and adipocytes, and may also make other non-neural cells such as cardiac muscle cells, pancreatic islet cells (e.g., alpha, beta, phi, delta cells), hematopoietic cells, hepatocytes, and the like. The cells may also be used for autologous or heterologous transplants to treat, for example, other neurodegenerative diseases, disorders, or abnormal physical states.

Accordingly, in a first aspect, the invention features MNSCs substantially purified from a peripheral tissue of a postnatal mammal. In one embodiment the peripheral tissue includes a sensory receptor.

In a second aspect, the invention features a cell that is the progeny of a MNSC substantially purified from a peripheral tissue of a postnatal mammal. The cell may be a mitotic cell or a differentiated cell (e.g., a neuron, an astrocyte, an oligodendrocyte, a Schwann cell, or a non-neural cell). Preferred neurons include neurons expressing one or more of the following neurotransmitters: dopamine, GABA, glycine, acetylcholine, glutamate, and serotonin. Preferred non-neural cells include cardiac muscle cells, pancreatic cells (e.g., islet cells (alpha, beta, phi and delta cells,)), exocrine cells, endocrine cells, chondrocytes, osteocytes, skeletal muscle cells, smooth muscle cells, hepatocytes, hematopoietic cells, and adipocytes.

In a third aspect, the invention features a population of at least ten cells, wherein at least 30% of the cells are MNSCs substantially purified from a peripheral tissue of a postnatal mammal or progeny of the MNSCs. In one embodiment the peripheral tissue includes a sensory receptor.

Preferably, at least 50% of the cells are MNSCs substantially purified from the peripheral tissue or progeny of the MNSCs. More preferably, at least 75% of the cells are MNSCs substantially purified from the peripheral tissue or progeny of the MNSCs. Most preferably, at least 90%, 95%, or even 100% of the cells are MNSCs substantially purified from the peripheral tissue or progeny of the MNSCs. The MNSCs may be cultured for extended periods of time. Thus, the population of cells may have been in culture for at least thirty days, sixty days, ninety days, or

longer (e.g., one year or more). Preferably, the population is at least twenty cells, and may be more than fifty cells, a thousand cells, or even a million cells or more.

In a fourth aspect, the invention features a pharmaceutical composition including (i) a mitotic or differentiated cell that is the progeny of a MNSC
5 substantially purified from a peripheral tissue of a postnatal mammal, and (ii) a pharmaceutically acceptable carrier, auxiliary or excipient.

In a fifth, related aspect, the invention features a pharmaceutical composition including (i) a MNSC substantially purified from a peripheral tissue of a postnatal mammal, and (ii) a pharmaceutically acceptable carrier, auxiliary or
10 excipient.

Preferably, the composition of the fourth or fifth aspect includes a population of cells, wherein at least 30%, 50%, 75%, 90%, 95%, or even 100% of the cells are MNSCs substantially purified from the peripheral tissue or progeny of the MNSCs. The composition may include one or more types of cells selected from
15 a group consisting of MNSCs, or neurons, oligodendrocytes, Schwann cells, astrocytes, adipocytes, smooth muscle cells, cardiomyocytes, chondrocytes, osteocytes, skeletal muscle cells, hepatocytes, hematopoietic cells, exocrine cells endocrine cells and alpha, beta, phi and delta cells, which are progeny of MNSCs.

In a sixth aspect, the invention features a method of producing a
20 population of at least ten cells, wherein at least 30% of the cells are MNSCs substantially purified from a peripheral tissue of a postnatal mammal or progeny of the MNSCs, wherein the peripheral tissue includes a sensory receptor, the method including: (a) providing the peripheral tissue from the mammal; (b) culturing the tissue under conditions in which MNSCs proliferate and in which at least 25% of the
25 cells that are not MNSCs die; and (c) continuing culture step (b) until at least 30% of the cells are MNSCs or progeny of the MNSCs.

In a seventh aspect, the invention features another method of producing a population of at least ten cells, wherein at least 30% of the cells are MNSCs substantially purified from skin tissue of a postnatal mammal or progeny of the

MNSCs, the method including: (a) providing the skin tissue from the mammal; (b) culturing the tissue under conditions in which MNSCs proliferate and in which at least 25% of the cells that are not MNSCs die; (c) separating the MNSCs from cells that are not MNSCs; and (d) repeating steps (b) and (c) until at least 30% of the cells
5 are MNSCs or progeny of the MNSCs.

Suitable culture conditions for step (b) of the sixth and seventh aspects are preferably as follows: (i) triturating or otherwise separating tissue into single cells or cell clusters and placing into culture medium; (ii) culturing the cells in culture medium and under conditions (*e.g.*, DMEM: Ham's F-12 medium containing B-27
10 supplement, antibacterial and antifungal agents, 5-100 ng/ml bFGF, and 2-100 ng/ml EGF) that allows for the proliferation of MNSCs but does not promote, to the same extent, proliferation of cells that are not MNSCs; and (iii) culturing the separated tissue for three to ten days, during which time the MNSCs proliferate in suspension but non-MNSCs do not proliferate in suspension (these cells either attach to the
15 plastic or they die). Preferably, at least 50% of the cells in suspension surviving after the period in culture are MNSCs or progeny of the MNSCs, more preferably, at least 75% of the cells are MNSCs or progeny of the MNSCs, and, most preferably, at least 90% or even 95% of the surviving cells are MNSCs or progeny of the MNSCs. In preferred embodiments tissue is separated mechanically.

20 In an eighth aspect, the invention features a method of treating a patient having a disease associated with cell loss. The method includes the step of transplanting the multipotent stem cells of the invention into the region of the patient in which there is cell loss. Preferably, prior to the transplanting step, the method includes the steps of providing a culture of peripheral tissue, preferably containing
25 sensory receptors, from the patient and isolating a multipotent neural stem cell from the peripheral tissue. After transplanation, the method may further include the step of differentiating (or allowing the differentiation of) the MNSCs into a desired cell type to replace the cells that were lost. Preferably, the region is a region of the CNS

or PNS, but can also be cardiac tissue, pancreatic tissue, or any other tissue in which cell transplantation therapy is possible.

In a ninth aspect, the invention features a kit including MNSCs substantially purified from peripheral tissue of a postnatal mammal, or a mitotic or differentiated cell that is the progeny of the MNSC, preferably wherein the peripheral tissue from which the MNSC is purified includes a sensory receptor. Preferably, the kit includes a population of cells, wherein at least 30%, 50%, 75%, 90%, or even 95% of the cells are MNSCs substantially purified from the peripheral tissue or progeny of the MNSCs.

In a tenth aspect, the invention features a kit for purifying MNSCs from peripheral tissue containing sensory receptors. The kit includes media or media components that allow for the substantial purification of MNSCs of the present invention. The kit may also include media or media components that allow for the differentiation of the MNSCs into the desired cell type(s). Preferably, the kit also includes instructions for its use.

In one preferred embodiment of each of the foregoing aspects of the invention, the peripheral tissue is skin tissue. In another preferred embodiment, the peripheral tissue is olfactory epithelium, tongue tissue, hair follicles, sweat glands, or sebaceous glands. In still another embodiment, the peripheral tissue of the first aspect specifically excludes olfactory epithelium and tongue tissue. In another preferred embodiment of each of the foregoing aspects of the invention, the stem cells do not express detectable levels of p75.

The peripheral tissue can be from a newborn mammal, a juvenile mammal, or an adult mammal. Preferred mammals include, for example, humans, non-human primates, mice, pigs, and rats. The MNSCs can be derived from peripheral tissue of any individual, including one suffering from a disease or from an individual immunologically compatible to an individual suffering from a disease. In a preferred embodiment, the cells, or progeny of the cells, are transplanted into the CNS or PNS of an individual having a neurodegenerative disease and the individual

is the same individual from whom the MNSCs were purified. Following transplantation, the cells can differentiate into cells that are lacking or non-functional in the disease.

Preferably, the MNSCs express nestin and fibronectin and may also
5 express glutamic acid decarboxylase. The MNSCs of the present invention can, under appropriate conditions, differentiate into neurons, astrocytes, Schwann cells, oligodendrocytes, and/or non-neural cells (e.g., cardiac cells, pancreatic cells, smooth muscle cells, adipocytes, hepatocytes, etc.).

MNSCs can be stably or transiently transformed with a heterologous gene
10 (e.g., one encoding a therapeutic protein, such as a protein which enhances cell divisions or prevents apoptosis of the transformed cell or other cells in the patient, or a cell fate-determining protein).

By “multipotential stem cell” or “multipotent neural stem cell” is meant a cell that (i) has the potential of differentiating into at least two cell types selected
15 from a neuron, an astrocyte, and an oligodendrocyte, and (ii) exhibits self-renewal, meaning that at a cell division, at least one of the two daughter cells will also be a stem cell. The non-stem cell progeny of a single MSC or MNSC are capable of differentiating into neurons, astrocytes, Schwann cells, and oligodendrocytes. Hence, the neural stem cell is “multipotent” because its progeny have multiple
20 differentiative pathways. The MSC or MNSC also has the potential to differentiate as another cell type (e.g., a skin cell, a hematopoietic cell, a smooth muscle cell, a cardiac muscle cell, a skeletal muscle cell, or a pancreatic cell). The term MSC or MNSC is not intended to be limited to cells that are developmentally derived from neural lineages.

25 By “substantially purified” is meant that the desired cells (e.g., MNSCs) are enriched by at least 30%, more preferably by at least 50%, even more preferably by at least 75%, and most preferably by at least 90% or even 95%.

By “therapeutic protein” is meant a protein that improves or maintains the health of the cell expressing the protein or a that of a cell in proximity to the

expressing cell. Example therapeutic proteins include, without limitation, growth factors (NGF, BDNF, NT-3, NT-4/5, HGF, TGF- family members, PDGF, GDNF, FGF, EGF family members, IGF, insulin, BMPs, Wnts, hedgehogs, and heregulins) cytokines (LIF, CNTF, TNFm interleukins, and gamma-interferon), and anti-
5 apoptotic proteins (IAP proteins, Bcl-2 proteins, Bcl-X_L, Trk receptors, Akt, PI3 kinase, Gab, Mek, E1B55K, Raf, Ras, PKC, PLC , FRS2, rAPs/SH2B, and Np73).

By “peripheral tissues” is meant a tissue that is not derived from neuroectoderm, for example peripheral tissue containing sensory receptors, and specifically includes olfactory epithelium, tongue, skin (including dermis and/or
10 epidermis), and mucosal layers of the body (e.g., mouth, reproductive system).

By a “population of cells” is meant a collection of at least ten cells. Preferably, the population consists of at least twenty cells, more preferably at least one hundred cells, and most preferably at least one thousand or even one million cells. Because of the MNSCs of the present invention exhibit a capacity for self-
15 renewal, they can be expanded in culture to produce populations even billions of cells.

By “postnatal” is meant an animal that has been born at term.

By “a disease characterized by failure of a cell type” is meant one in which the disease phenotype is the result of loss of cells of that cell type or the loss
20 of function of cells of that cell type.

Other features and advantages of the present invention will become apparent from the following detailed description and the claims. It will be understood, however, that the detailed description and the specific examples, while indicating preferred embodiments of the invention, are given by way of example
25 only, and various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

Brief Description of the Drawings

Figs. 1A-1G are photographs showing that mouse skin-derived MNSCs are nestin-positive and are capable of differentiating into neurons, glia, and smooth muscle cells.

Fig. 2 is a series of photographs showing that neonate and adult mouse skin-derived MNSCs express both nestin (middle row) and fibronectin (bottom row).

Fig. 3A is a series of photographs showing western blot analysis for nestin, neurofilament M (NF-M) and GFAP in cells differentiated from neonate and adult mouse skin-derived MNSCs.

Fig. 3B is a series of photographs showing that human skin-derived MNSCs express nestin.

Fig. 3C is a series of photographs showing that a subset of morphologically complex cells expressed nestin and -tubulin, a profile typical of newly-born neurons.

Fig. 3D is a series of photographs showing that GFP positive cells are also positive for neuron-specific enolase.

Fig. 4A is a photograph showing the expression of A2B5, a marker for oligodendrocyte precursors, on undifferentiated mouse skin-derived MNSCs.

Fig. 4B is a photograph showing the expression of the oligodendrocyte marker galactocerebroside (GalC) on cells differentiated from mouse skin-derived MNSCs.

Fig. 5 is a series of photographs showing that the fate of mouse skin-derived MNSCs can be manipulated by controlling plating conditions.

Fig. 6 is a series of photographs showing that neonate and adult mouse skin-derived MNSCs can differentiate as adipocytes.

Figs. 7A and 7B are photographs showing that nestin-positive, fibronectin-positive MNSCs can be derived from mouse dermis.

Figs. 8A and 8B are photographs showing that individual MNSCs are multipotent. Clones derived from single cells contained NF-M-positive cells (arrowheads) and CNPase-positive cells (arrows) (Fig. 3A). In Fig. 3B, arrowheads

indicate cells that only express GFAP, while arrows indicate cells expressing both GFAP and CNPase.

Figs. 9A and 9B are photographs of western blot analysis of cells differentiated from mouse skin-derived MNSCs (Fig. 9A) or of MNSCs themselves (Fig. 9B).

Fig. 10 is a series of photographs showing the effect of various pharmacological agents on mouse skin-derived MNSCs.

Figs. 11A-11E are photographs of immunoprocessed sections of rat brains into which mouse skin-derived MNSCs were transplanted.

Detailed Description of the Invention

We have substantially purified multipotent stem cells, e.g., neural stem cells (MNSCs), from peripheral tissues of mammals, including skin, olfactory epithelium, and tongue. These cells proliferate in culture, so that large numbers of stem cells can be generated. These cells can be induced to differentiate, for example, into neurons, astrocytes, and/or oligodendrocytes by altering the culture conditions. They can also be induced to differentiate into non-neural cells such as smooth muscle cells and adipocytes. The substantially purified neural stem cells are thus useful for generating cells for use, for example, in autologous transplants for the treatment of degenerative disorders or trauma (*e.g.*, spinal cord injury). In one example, MNSCs may be differentiated into dopaminergic neurons and implanted in the substantia nigra or striatum of a Parkinson's disease patient. In a second example, the cells may be used to generate oligodendrocytes for use in autologous transplants for the treatment of multiple sclerosis. In still another example, the MNSCs may be used to generate Schwann cells for treatment of spinal cord injury, cardiac cells for the treatment of heart disease, or pancreatic islet cells for the treatment of diabetes. If desired, in any of the foregoing examples, the cells may be genetically modified to express, for example, a growth factor, an anti-apoptotic protein, or another therapeutic protein.

The MNSCs display some similarities to stem cells derived from mammalian forebrain, but also possess some distinctive differences. In particular, when the MNSCs of the present invention differentiate in the presence of serum, about 5-20% of the differentiated cells express neuronal markers, whereas
5 differentiated forebrain stem cells generate only a small percentage of neurons. Moreover, significant numbers of dopaminergic neurons are found in differentiated cultures of MNSCs of the present invention, whereas such neurons have not been observed in cultures of forebrain stem cells differentiated in serum.

10

Cell Therapy

The multipotent stem cells of this invention may be used to prepare pharmaceutical compositions that can be administered to humans or animals for cell therapy. The cells may be undifferentiated or differentiated prior to administration.
15 Dosages to be administered depend on patient needs, on the desired effect, and on the chosen route of administration.

The invention also features the use of the cells of this invention to introduce therapeutic compounds into the diseased, damaged, or physically abnormal CNS, PNS, or other tissue. The MNSCs thus act as a vector to transport
20 the compound. In order to allow for expression of the therapeutic compound, suitable regulatory elements may be derived from a variety of sources, and may be readily selected by one with ordinary skill in the art. Examples of regulatory elements include a transcriptional promoter and enhancer or RNA polymerase binding sequence, and a ribosomal binding sequence, including a translation
25 initiation signal. Additionally, depending on the vector employed, other genetic elements, such as selectable markers, may be incorporated into the recombinant molecule. The recombinant molecule may be introduced into the stem cells or the cells differentiated from the stem cells using *in vitro* delivery vehicles such as retroviral vectors, adenoviral vectors, DNA virus vectors and liposomes. They may

also be introduced into such cells *in vivo* using physical techniques such as microinjection and electroporation or chemical methods such as incorporation of DNA into liposomes. The genetically altered cells may be encapsulated in microspheres and implanted into or in proximity to the diseased or damaged tissue.

5 In one embodiment, the MNSCs are used for the treatment of neurological disease. In another aspect the MNSCs of the present invention may also be used as a source of non-neural cells, for example adipocytes and smooth muscle cells. As an example, PCT publication WO99/16863 describes the differentiation of forebrain MNSCs into cells of the hematopoietic cell lineage *in vivo*. The MNSCs of the
10 present invention appear to be more plastic and thus are highly likely to also be capable of differentiating into non-neural cells types, such as hematopoietic cells. Accordingly, the invention features methods of treating a patient having any disease or disorder characterized by cell loss by administering MNSCs of the present invention (or cells derived from these cells) to that patient and allowing the cells to
15 differentiate to replace the cells lost in the disease or disorder. For example, transplantation of MNSCs and their progeny provide an alternative to bone marrow and hematopoietic stem cell transplantation to treat blood-related disorders. Other uses of the MNSCs are described in Ourednik et al. (Clin. Genet. 56:267-278, 1999), hereby incorporated by reference. MNSCs and their progeny provide, for example,
20 cultures of adipocytes and smooth muscle cells for study in vitro and for transplantation. Adipocytes secrete a variety of growth factors that may be desirable in treating cachexia, muscle wasting, and eating disorders. Smooth muscle cells may be, for example, incorporated into vascular grafts, intestinal grafts, etc.

25 Example 1: Purification of MNSCs from postnatal mouse olfactory epithelium

 MNSCs from mouse olfactory epithelium were purified as described below. Postnatal mice were stunned with a blow to the head and then decapitated. The heads were sagittally sectioned with a razor blade, and the olfactory epithelia of about six postnatal (P1-P9) mouse pups were stripped from the conchae, nasal

septum, and vomeronasal organs using watch-maker forceps. This tissue was placed into 3 mL of medium (DMEM/F-12 3:1) supplemented with 2% B-27 (Gibco, Burlington, Ontario, Canada), 20 ng/mL epidermal growth factor (EGF; Collaborative Research, Bedford, MA), 0.1% fungizone, and 0.5 mL/100mL penicillin/streptomycin (Gibco). Following collection, the epithelia were teased apart with watchmaker forceps, releasing a large number of single cells and small cell clusters. The cell suspension was transferred to a 15 mL tube, and 7 mL of additional medium was added. The clusters were dissociated into single cells by titration with a 10 mL plastic pipette and passed through a 60 micron filter (Gibco). Typically, dissociated cells from the olfactory epithelia from six pups were plated into two 50 mL tissue culture flasks and cultured in a 37 C, 5% CO₂ tissue culture incubator. Two days later, most cells in the cultures were dead or dying. A small number (less than 1% of the initial cell number) of large, phase bright cells were present, however, most of which were attached to the flask bottom. Over the next two to six days, these cells divided and produced spherical clusters, which became larger over time. At four to five days in culture, there were approximately 500 clusters of dividing cells per pup used in the original purification. Most of these cell clusters detached from the flask surface over the next few days. These detached cell clusters continued to grow and fused together to become macroscopic, reaching approximately 100 µm in diameter following 10 DIV. After 12 DIV, the cell clusters became macroscopic, reaching approximately 200 µm or greater in diameter.

If EGF was not added to the medium, small clusters of dividing cells were still seen by 4 DIV, and some cell were formed, indicating that the cells themselves were producing trophic factors in quantities that, in some cases, was sufficient to maintain their proliferation.

Greater than 95% of the cells in the dividing clusters expressed nestin, a marker for stem cells and neural stem cells. These nestin-positive cells could be repeatedly passaged, indicating that the cells were neural stem cells. Six days after

purification, the medium (5 mL) was removed from the flasks. This medium contained many clusters of stem cells that had detached from the flask surface. The detached cells were triturated with a fire-polished pipette, thereby dissociating many of the cell clusters into single cells. The medium containing the cells was then
5 placed in a second flask with an additional 15 mL of fresh medium (total volume = 20 mL). After a further six days, one quarter of the medium was removed and the detached clusters of cells were again triturated and transferred to a new flask with 15 mL fresh medium. These cells have been successfully passaged more than twenty times without losing their multipotency.

10

Example 2: Differentiation of mouse MNSCs into neurons, astrocytes and oligodendrocytes

After the cellular clusters of Example 1 had been generated, they could be differentiated into neurons, astrocytes, and oligodendrocytes. Clusters from cultures
15 7 to 14 days after purification were plated onto polylysine coated 35 mm culture dishes or 4 multiwell culture dishes, in DMEM/F12 media containing 2% fetal bovine serum (Hyclone, Logan, UT) and 2% B-27 (containing no EGF). The medium was changed every three to four days. Over the next six to nineteen days, cells migrated out of the clusters onto the dish surface. Some of these cells had the
20 morphology of neurons, astrocytes, or oligodendrocytes. We determined the phenotype of these cells using the following antibodies: GFAP for astrocytes; neurofilament 160 (NF-160), MAP-2, III tubulin, and NeuN for neurons; and GC for oligodendrocytes. Antibodies to TH were used to identify dopaminergic, noradrenergic, and adrenergic neurons. Dopamine -hydroxylase (DBH) was also
25 used for noradrenergic and adrenergic neurons.

Astrocytes, neurons, and oligodendrocytes were all found to differentiate from the MNSCs of this invention, indicating that the cells were multipotent. We also cultured MNSCs from transgenic mice which express -galactosidase off of the neuron specific T 1 -tubulin promoter, which allowed us to use staining with the

ligand X-gal antibodies for -galactosidase as an additional neuronal marker. We observed -galactosidase-positive cells.

Since the majority of differentiated cells remained in clusters, it was not possible to determine the percentage of cells expressing each marker. The majority of cells that migrated out of the clusters were GFAP positive, while a large number of cells were either NeuN or -galactosidase positive. A lower number of cells were GC positive. Therefore the MNSCs could differentiate into neurons, astrocytes and oligodendrocytes. TH-positive cells were also identified. These TH-positive cells are most likely dopaminergic neurons and not noradrenergic or adrenergic neurons, since no cells were found to be DBH positive. Significantly, no TH, GFAP or GC positive cells have ever been reported *in vivo* in the nasal epithelium. Therefore the olfactory epithelium-derived nestin-positive MNSCs are capable of differentiating into cell types (e.g., oligodendrocytes, astrocytes, GABAergic neurons, and dopaminergic neurons) never found in the olfactory epithelium.

Like the originally-purified olfactory MNSCs, MNSCs passaged from two to twenty times could also differentiate into neurons, astrocytes, and oligodendrocytes. MNSCs which had been passaged were plated on polylysine-coated dishes. Cells migrated from the clusters and spread out over the surface of the dish. After 16 DIV, cells that were immunopositive for GC, GFAP, III tubulin, NeuN, lacZ, or TH could be identified. Moreover, the proportion of cells positive for the various markers was similar to that seen in the differentiated cultures from the original cultures.

Example 3: Purification of MNSCs from olfactory epithelial tissue of adult mice and rats

Similar to the foregoing results, MNSCs were also purified from adult mouse and rat olfactory epithelium and vomeronasal organ using the methods described in Examples 1 and 2.

Adult mice and rats were anaesthetized with an overdose of somnitol, and then decapitated. The olfactory and vomeronasal organ epithelia were stripped from the conchae and nasal septum and incubated in DMEM/F12 medium for one to two days after their dissection and prior to the rest of the purification procedure. After this incubation, the epithelia were dissociated in an identical manner as the epithelia from juvenile mice. Two days after the isolation, the majority of the cells were dead with the exception of a very few large phase bright cells. These cells divided over the next few days, forming small clusters of dividing cells similar to those described in Example 1. These small clusters grew to give rise to the large clusters that detached from the culture dish surface. After approximately six divisions, cells in some of these clusters began to differentiate and spread out over the flask's surface, while some other clusters, which had been floating, reattached to the surface and then produced differentiated cells. In some cases, cells multiplied to produce small clusters of cells, but did not grow to form large cell clusters like the postnatal cultures. We have passaged these cells twenty times using the same procedure as that described above with respect to the cells purified from juvenile olfactory epithelium. These proliferating cells from the adult were also nestin-positive.

After the cell clusters derived from adult tissue had been generated, the cells could be differentiated into neurons, astrocytes, and oligodendrocytes. Seven days after isolation, clusters were plated onto polylysine-coated 35 mm culture dishes or multi-well culture dishes, in medium containing 2% fetal bovine serum and 2% B-27, but no EGF. Over the next month, cells migrated from the cell clusters and onto the dish surface. We determined the phenotype of these cells using antibodies to astrocytes, neurons, dopaminergic neurons, and oligodendrocytes as described above.

Neurons (including dopaminergic neurons), astrocytes, and oligodendrocytes were found, although the number of these cells was much lower than the number obtained from the juvenile. The cells purified from adult olfactory epithelia are self-renewing and multipotent, and thus are MNSCs.

Example 4: Purification of MNSCs from mouse tongue

We derived MNSCs from the tongue, another peripheral tissue that contains sensory receptors. The tongue was dissected to remove the epithelial layer that contains the sensory receptors and their underlying basal cells. This layer of tissue was triturated to produce single cells and the single cells were plated in flasks containing DMEM/F12 media supplemented with B-27 and EGF, TGF , and/or bFGF, as described for the olfactory epithelium. After two to three days in a 37°C, 5% CO₂ tissue culture incubator, greater than 99% of the cells in the culture were dead or dying. A small number (less than 1%) of large phase-bright cells were present, however, most of which attached to the flask bottom. Over the next two to six days, these cells divided and produced spherical clusters that became larger over time and detached from the flask surface. The cells in these clusters were nestin-positive.

These nestin-positive MNSCs can be passaged using the same techniques as used for the multipotent stem cells derived from the olfactory epithelium. Similarly, the MNSCs can be differentiated into neurons, astrocytes and oligodendrocytes using the techniques described herein.

Example 5: Purification of MNSCs from mouse skin

Skin from neonatal mice aged 3-15 days was dissociated and cultured in uncoated flasks containing 20 mg/mL EGF and 40 mg/mL bFGF. Over the subsequent one to five days, many (>90%) of the cells die. A small population of cells hypertrophy and proliferate to form small cell clusters growing in suspension. Some of these cells first attach to the tissue cluster plastic, hypertrophy and proliferate, and then detach as the clusters become of sufficient size. Other cells never attach to the tissue culture plastic and instead proliferate in suspension from the beginning. After four to five days, the cell clusters are small but easily distinguishable as clusters of proliferating cells. By seven to ten days, many of the

cell clusters reach diameters of as much as 100 μm , while by two weeks, the cell clusters are macroscopic if left unperturbed. Many cells adhered to the plastic, and many died, but by about three to seven days, suspended clusters of up to about 20 cells formed. These suspended or floating cells were transferred to a new flask seven days after initial culturing; again, many cells adhered, but the cells in the floating clusters proliferated to generate larger clusters of more than about 100 cells (Fig. 1A, top panel). These larger clusters were then isolated, dissociated and passaged. By this process of selective adhesion, substantially pure populations of floating clusters were obtained after 3 to 4 weeks. Cells that generated these clusters were relatively abundant; 1.5 to 2 cm^2 of abdomen skin was sufficient to generate six 25 cm^2 flasks of floating clusters over this period of time.

To determine whether clusters contained MNSCs, we dissociated the clusters and plated the cells onto poly-D-lysine/laminin-coated dishes or chamber slides without growth factors and, 12 to 24 hours later, immunostained them for the presence of the neural precursor-specific marker nestin. After three passages, the majority of the cells expressed nestin (Fig. 1B, top panel), a property they maintained over subsequent passages. They did not, however, express the p75 neurotrophin receptor, a marker for neural crest stem cells, as detected either by immunocytochemistry or western blots.

We also determined whether the skin-derived MNSCs expressed fibronectin. Four lines of skin-derived MNSCs cultured from either adult (Fig. 2; left two columns) or neonatal (right two columns) mouse skin, cultured for either long term (first and third columns) or short term (second and fourth columns) were each dissociated, plated for two days in DMEM/F12 (3:1) containing 2% B-27 supplement, and then immunostained for nestin and fibronectin. As is demonstrated in Fig. 2, the majority of cells expressed both markers.

To determine whether clusters of cells could be generated from adults, skin of adult mice was dissociated and cultured as described above. Similar to neonatal mouse skin, most cells adhered to the flask or died when first cultured.

After three to seven days, however, clusters of up to approximately 20 cells were observed that subsequently increased in size. When these cells were passaged at least three times (Fig. 1A, bottom panel), and plated onto poly-D-lysine/laminin overnight in the absence of growth factors, they too were immunopositive for nestin (Fig. 1B, bottom panel) and fibronectin (Fig. 2). The nestin-positive cells from adults and neonates have been passaged in this manner for over 30 passages, during which time the number would have theoretically expanded at least 10^9 -fold (assuming a doubling time of approximately one week).

To determine whether these nestin-positive, fibronectin-positive cells from skin could generate neural cell types, we analyzed neonatal skin-derived cells after three or more passages and greater by plating them on poly-D-lysine/laminin in the absence of growth factors. Immunostaining (Figs. 1C and 1D) and western blot analysis (Fig. 3A) revealed that the skin-derived cells expressed neuronal markers. At seven days, a subpopulation of morphologically-complex cells coexpressed nestin and neuron-specific β III-tubulin, a profile typical of newly-born neurons (Fig. 1C). At later time points of 7-21 days, cells also expressed neurofilament-M (NF-M)(Figs. 1D, 3A) and neuron-specific enolase, two other neuron-specific proteins. Finally, some neurofilament-positive cells expressed GAD (Fig. 1D), a marker for GABAergic neurons, which are not found in the PNS. Similar results were obtained for adult skin-derived MNSCs, although at early passages some of the the β III-tubulin and neurofilament-positive cells were less typically neuronal in morphology.

Immunostaining and western blots revealed that both neonatal and adult MNSCs generated cells expressing the glial markers GFAP and CNPase at seven to twenty-one days after plating (Figs. 1D-1F, 2A). Double-labeling for these proteins demonstrated the presence of (i) cells that were GFAP- positive but not CNPase-positive (potentially astrocytes), (ii) cells that expressed CNPase but not GFAP (potentially oligodendrocytes or their precursors), and (iii) a small subpopulation that were bipolar and expressed both CNPase and GFAP (potentially Schwann cells) (Fig. 1E). A subpopulation of GFAP-positive cells also expressed nestin, a finding

previously reported for developing CNS astrocytes. Additionally, some cells were positive for A2B5, a marker for oligodendrocyte precursors (Fig. 4)). Like GAD-positive neurons, astrocytes and oligodendrocytes are normally found only in the CNS.

5 Double-labeling studies supported the following additional conclusions. First, glial versus neuronal markers were expressed in distinct subpopulations of MNSCs progeny. Second, after twenty passages, skin-derive MNSCs were still able to differentiate into neurons and glial cells. Finally, skin-derived MNSCs were able to generate smooth muscle cells (as determined by both expression of smooth
10 muscle actin (SMA) and morphology; Fig. 1G) and adipocytes (Figs. 5 and 6).

Example 6: MNSCs originate from the dermal layer of the skin

 The two major layers of the skin are the epidermis and the dermis. To determine the origin of the skin-derived MNSCs, we dissected and cultured P7, P14,
15 and P18 mouse epidermis and dermis. The two layers of the skin were separated by incubating the skin pieces (1 X 2 cm²) in 0.2% trypsin at 40°C for about 24-36 hours, or until the dermis could be separated from the epidermis. The cells in each layer were dissociated separately and then cultured in DMEM/F12 (3:1) with B-27 supplement, EGF (20 ng/mL) and FGF (40 ng/mL). Only the cells derived from the
20 dermis generated cluster of cells similar to those derived from whole skin (Fig. 7A). No viable cells were obtained from the epidermis. To characterize the dermis-derived cell clusters, the clusters were cultured for four weeks and then plated onto tissue culture chamber slides coated with poly-D-lysine and laminin. After 24 hours, the cells were then processed for immunocytochemistry. Like MNSCs derived from
25 whole mouse skin, the dermis-derived cells coexpressed nestin and fibronectin (Fig. 7B).

Example 7: Clonal analysis indicates that skin-derived MNSCs are multipotent

To determine whether skin-derived MNSCs are multipotent, we isolated single cells by limiting dilution of cells from clusters that three months prior had been derived from neonatal mice. We cultured the cells for five weeks in medium from the same culture line and containing growth factor, and then differentiated the cells for two weeks in medium lacking growth factor but containing 3% rat serum. The cells were then processed for immunocytochemistry. As is demonstrated in Fig. 8, single clones of cells contained NF-M- and CNPase-positive cells (Fig. 8A), and GFAP- and CNPase-positive cells (Fig. 8B).

10 Example 8: Western blot analysis of skin-derived MNSCs

For western blot analysis of skin-derived MNSCs, four cultures (one adult-derived line and three neonate-derived lines) that had been passaged from seven to 40 times were analyzed either as clusters or following differentiation by plating in medium containing 1% FBS, B-27 supplement, and fungizone for 14 days in 60 mm dishes coated with poly-D-lysine and laminin. Cell lysates were prepared, and equal amounts (50-100 µg) of protein from each culture were separated on 7.5% or 10% polyacrylamide gels, transferred to membrane, and then probed with anti-nestin monoclonal antibody (1:1000; Chemicon), anti NF-M polyclonal antibody (1:1000; Sigma), anti GFAP polyclonal antibody (1:1000, Dako), or antifibronectin polyclonal antibody (1:1000; Sigma). As positive controls, we used cortical progenitor cells cultured in the presence of CNTF (which results in astrocytic differentiation) or in the absence of CNTF (which results in neuronal differentiation) and adult mouse cortex. As negative controls, we used sympathetic neurons and liver. As illustrated in Fig. 9A, western blotting confirmed the expression of GFAP and NF-M in cultures differentiated from both adult and neonate skin-derived MNSCs. Similarly, Fig. 9B illustrates the expression of both nestin and fibronectin in adult and neonate skin-derived MNSC clusters.

25 Example 9: MNSC differentiation can be modulated by plating conditions

As is illustrated above, when clusters of skin-derived MNSCs are dissociated and plated in medium containing FGF and EGF, most of the nestin-positive cells become neurofilament-positive. We have found that when the cells are plated in medium containing 10% FBS, the cells adopt a morphology similar to that displayed by adipocytes. The adoption of the adipocyte cell fate was confirmed by staining with Oil Red O (Fig. 5). The ability of 10% FBS to induce adipocyte differentiation was true for both adult and neonate skin-derived MNSCs (Fig. 6).

Example 10: Pharmacological inhibitors affect survival and proliferation of skin-derived MNSCs

When skin-derived MNSCs are plated for three days in proliferation medium containing FGF, they typically exhibit a spherical morphology characteristic of their proliferative state (Fig. 10). We tested the ability of pharmacological agents to alter this phenotype. Supplementing the medium with PD098059 (an inhibitor of the ERK MAPK pathway) caused proliferating cells to flatten and differentiate (Fig. 10), while supplementing with LY294002 (an inhibitor of the PI-3-K pathway, caused the cells to die (Fig. 10). The p38 MAPK inhibitor SB203580 had no observed effect on the proliferating skin-derived MNSCs.

Example 11: Purification of nestin-positive cells from adult human skin

We have purified nestin-positive cells from human scalp. To purify MNSCs from human skin, we utilized tags of scalp tissue generated by placement of a stereotactic apparatus during neurosurgery. Scalp tags totalling 1 cm² or less from each of eight individuals were used. The skin included dermal and epidermal tissue. Tissue was cut into smaller pieces that were then transferred into HBSS containing 0.1% trypsin for forty minutes at 37°C. Following trypsinization, tissue samples were washed twice with HBSS and once with DMEM:F12 (3:1) supplemented with

10% rat serum to inactivate the trypsin. Trypsinized tissue was then mechanically dissociated by trituration in a pipette and the resulting dispersed cell suspension was poured through a 40 µm cell strainer into a 15 mL tube. The tube was then centrifuged for five minutes at 1000 rpm (~1200 X g). The cells were resuspended in DMEM:F12 medium containing 40 ng/mL bFGF, 20 ng/mL EGF, 2% B-27 supplement, and antibacterial and antifungal agents, and then cultured in 12 well plastic tissue culture plates. Every seven days, the cell clusters are harvested by centrifugation, triturated with a fire-polished pasteur pipette, and cultured in fresh medium.

As for the use of rodent skin, most cells (>75%) adhered to the plastic or died, but after seven days, small floating clusters of cells were observed. These clusters were then partially dissociated and transferred to new wells, where they slowly increased in size. After additional passaging, clusters were plated on poly-D-lysine/laminin in 3% FBS with no growth factors, and analyzed for the presence of neural markers.

Within two weeks, greater than 30% of the cells within the cell clusters were nestin-positive (Fig. 3B). Immunolabeling of four to six week old cultures also revealed that many of the cells in the clusters were nestin-positive with the percentage varying from less than 50% to greater than 80% two to three days after plating. Double-label immunocytochemistry at the same or longer time-points revealed that, in all cultures, some nestin-positive cells also expressed βIII-tubulin and displayed elongated neurites (Fig. 3C). Thus, adult human skin is a source for nestin-positive MNSCs cells that, when differentiated, can express neuron-specific proteins.

Example 12: Purification and differentiation of MNSCs derived from other human peripheral tissues containing sensory receptors

MNSCs can be purified from human olfactory epithelium using the same procedures as described for the purification of stem cells from rodent olfactory epithelium. Source material is acquired by surgical removal of olfactory epithelial

tissue from the donor. Because the MNSCs are capable of proliferation and self-renewal, little source tissue is required. Preferably, the amount is at least about 1 mm³. Conditions for culturing human cells are described in Example 6, above. Other conditions are known to those skilled in the art, and can be optimized for proliferation or differentiation of neural stem cells, if desired.

We can purify MNSCs from other peripheral tissues containing sensory receptors, other than the olfactory epithelium, tongue, and skin, using techniques described herein. Passaging and differentiation of these cells is also performed using the same techniques described herein. Other peripheral tissues containing sensory receptors include, for example, mucosal membranes from the mouth or reproductive system.

Example 13: Transformation of MNSCs

In therapy for neurodegenerative diseases, it may be desirable to transplant cells that are genetically modified to survive the insults that caused the original neurons to die. In addition, MNSCs may be used to deliver therapeutic proteins into the brain of patients with neurodegenerative disorders to prevent death of host cells. Exemplary therapeutic proteins are described herein. In still another example, MNSCs can be induced to differentiate into a desired cell type by transforming the cells with nucleic acid molecules encoding proteins that regulate cell fate decisions (*e.g.*, transcription factors such as Isl-1, en-1, en-2 and nurr-1, implicated in regulating motorneuron and striatal phenotypes). Using such a method, it is possible to induce the differentiation of the specific cell types required for transplant therapy. Therefore, it would be advantageous to transform MNSCs with nucleic acid molecules encoding desired proteins. We have previously used recombinant adenovirus to manipulate both postmitotic sympathetic neurons and cortical progenitor cells, with no cytotoxic effects. We now have established that olfactory epithelial-derived MNSCs and skin-derived MNSCs can each be successfully transfected with high efficiency and low toxicity.

Example 14: Differentiation of MNSCs into the appropriate cell type *in vivo* following transplantation into adult rodent brain

One therapeutic use for the MNSCs of the present invention is autologous
5 transplantation into the injured or degenerating CNS or PNS to replace lost cell
types and/or to express therapeutic molecules. We demonstrate below that the
MNSCs can differentiate into neurons when transplanted into the adult brain.

If desired, the dopaminergic innervation of the adult striatum can be
unilaterally destroyed by a local infusion of 6-hydroxydopamine under conditions in
10 which noradrenergic neurons are spared. Several weeks later, MNSCs are
transplanted into both the intact and lesioned striatum. Alternatively, the cells can be
transplanted into unlesioned animals. The fate of the transplanted MNSCs is then
determined by immunohistochemistry. Exemplary transplantation studies are
described below. These studies demonstrate that transplanted MNSCs can
15 differentiate into neurons *in vivo*, as they can *in vitro*. In the former case,
differentiation and cell fate choice is controlled by the local environment into which
each cell is placed. Both *in vitro*-differentiated and undifferentiated cells are useful
therapeutically in the treatment, for example, of neurodegenerative disease (*e.g.*,
Parkinson's disease and multiple sclerosis) or spinal cord injury. For example,
20 dopaminergic neurons differentiated from MNSCs, or the MNSCs themselves, may
be transplanted into the substantia nigra or the striatum of patients having
Parkinson's disease. If desired, the MNSCs may also be genetically-modified to
express a desired protein.

In one example, the dopaminergic innervation to adult rat striatum was
25 first unilaterally lesioned with the chemotoxin 6-hydroxydopamine, and the efficacy
of the lesions was tested two weeks later by amphetamine-induced rotational
behavior. Two days prior to transplantation, rats were immunosuppressed with
cyclosporin. MNSCs, produced from olfactory epithelia as described herein, were
then stereotactically injected into the caudate-putamen complex on both the lesioned

and unlesioned sides. Sixteen days following transplantation, animals were sacrificed, and sections of the striatum were analyzed for nestin- and TH-immunoreactivity. Five of eight animals received successful injections of MNSCs in the striatum. Of these, four animals showed evidence of a nestin-positive tract on both the lesioned and unlesioned sides, although tracts on the lesioned side appeared to be more intensely nestin-immunoreactive. On adjacent sections, TH-positive cells were observed confined to an area close to the transplant tract on both the lesioned and unlesioned side. As many as 25-30 TH-positive cells were identified on each section. Cell morphology varied from small, round cells lacking processes to neurons that were morphologically complex with multiple fine processes. In some cases, the processes of these TH-positive neurons extended into the striatum for distances of up to 300 μm .

To confirm that these TH-positive neurons derived from the MNSCs, we performed two sets of experiments in which the transplanted cells were detectably-labeled. In one set of experiments, transplanted MNSCs were derived from T1:nlacZ transgenic mice, in which the neuron-specific T1 α -tubulin promoter drives expression of a nuclear-localized β -galactosidase marker gene.

Immunohistochemical analysis of animals receiving the transgenic MNSCs revealed the presence of β -galactosidase-positive neurons within the transplant tract, confirming that the transplanted MNSCs generated neurons *in vivo*, as they did *in vitro*. In a second set of experiments, MNSCs were labelled with BrdU for 18 hours, washed to remove the BrdU label, and then transplanted unilaterally into the 6-hydroxydopamine-lesioned striatum of animals (10 rats, 4 mice) prepared as described herein. Immunohistochemical analysis with anti-BrdU revealed that all animals showed evidence of BrdU-positive transplant tracts. Immunocytochemistry with anti-GFAP revealed that, in both xenografts and allografts, GFAP-positive cells with heterogeneous morphology were concentrated at the transplant site, but were also present in moderate amounts over the entire ipsilateral hemisphere, with additional scattered reactive astrocytes seen in the contralateral hemisphere.

GFAP-BrdU double-labelled cells were present mainly within or close to the transplant tract, and varied in morphology from small, round cells with only a few processes, to large polygonal or fusiform cells with multiple processes.

Immunohistochemistry with anti-TH revealed that TH-BrdU double-labeled cells were also present, although these were few in number relative to GFAP-BrdU positive cells. BrdU-TH double-labeled cells were mainly small to medium-sized without processes, although some examples of double-labeled cells with processes were found within and adjacent to, the transplant tract. Thus, MNSCs generated astrocytes and neurons *in vivo*, and a subpopulation of the latter were TH-positive.

Together, these findings show that peripheral tissue-derived MNSCs are capable of generating cell types that are never found within olfactory tissue, including oligodendrocytes and TH-positive neurons.

To determine whether skin-derived MNSCs also generate differentiated neural cell types *in vivo*, we tagged adult mouse skin-derived MNSCs with (i) BrdU, and (ii) a recombinant adenovirus expressing GFP, and then transplanted them as cell clusters of about 20 to about 100 cells into the lateral ventricles of P2 rats.

Immunostaining fourteen days later revealed that, in all animals analyzed (n=8), transplanted cells had migrated extensively (Fig. 11A). In particular, tagged cells had integrated into the cortex, the hypothalamus and the amygdala in all, and into the hippocampus in two of the transplanted brains (Fig. 11A). In the cortex, GFP-positive cells were located in patches (Figs. 11A, 11B) or occasionally as single cells (Fig. 11C), including some that had integrated into and adopted the morphology of layer V pyramidal neurons (Figs. 11B, 11C). These cells had triangular-shaped soma, and projected a presumptive apical dendrite from layer V towards layer I, in a manner similar to the endogenous layer V neurons. That these cells were neurons was demonstrated by double-labeling for neuron-specific enolase (Fig. 3D).

Immunocytochemical analysis also confirmed that these were transplanted cells, as BrdU-positive cells were present in the same locations as GFP-positive cells in all brains (Fig. 11B).

In both the amygdala and hippocampus, transplanted cells also displayed neuronal morphology. In the amygdala, GFP and BrdU-positive cells were large, with prominent nuclei, and extensive processes (Fig. 11E). In the hippocampus, transplanted cells had integrated into both the dentate gyrus and pyramidal cell layers, and their morphology was typical of the endogenous granule and pyramidal cells, respectively (Figs. 11A, 11E). GFP-positive staining was also seen within the molecular layer. Finally, GFP- and BrdU-positive cells were observed in other locations, such as the hypothalamus, where the morphology of many cells was not typically neuronal.

Skin-derived MNSCs transplanted into adult rats also survive and integrate. We labeled adult mouse skin-derived MNSCs that had been passaged more than thirty times with the nuclear dye 33258, washed extensively, and then injected the cells stereotactically into the brains of adult rats that were immunosuppressed with cyclosporin. Four weeks later, we sacrificed the animals by perfusion and processed the brains for histological examination. Hoeschst-labeled cells were present in the hippocampus, olfactory bulb, and striatum. From these data, we conclude that the transplanted skin-derived MNSCs are capable of survival following transplantation. Moreover, cells are capable of migrating from the site of injection to numerous brain regions.

Skin-derived MNSCs are also capable of survival, migration, and integration following transplantation into a hemisected adult mouse spinal cord. In this example, the cells were injected into the injured sides of hemisected spinal cords. Eight days later, the animals were sacrificed and the spinal cords processed for histological analysis. Hoechst-labeled cells were present at the site of the initial injection, and had also migrated extensively into the injured spinal cord.

Example 15: Differentiation of non-neural cells from MNSCs

In addition to being capable of differentiating as neural cells (*i.e.*, neurons, oligodendrocytes, astrocytes, and Schwann cells), the peripheral tissue-derived MNSCs are capable of differentiating as non-neural cells that are normally not found in the tissue from which the cells were derived. For example, we have demonstrated that the skin-derived MNSCs can differentiate as smooth muscle cells and adipocytes. It is likely that the cells described herein have even greater potential. Conditions for the differentiation of the MNSCs into smooth muscle cells is described herein.

Signals or conditions sufficient for inducing MNSCs to differentiate as other cell types (*e.g.*, lymphocytes, cardiac muscle cells, skeletal muscle cells, melanocytes, and pancreatic cells) are known in the art. For example, unique signals induce neural crest-derived stem cells to become melanocytes, cartilage, smooth muscle cells, or bone (for review, see LaBonne and Bronner-Fraser, *J. Neurobiol.*, 36:175-189, 1998; Sieber-Blum, *Intl. Rev. Cytol.* 197:1-33, 2000). Conditions for inducing CNS-derived neural stem cells to differentiate as non-neural cells such as smooth muscle cells, skeletal muscle cells, hepatocytes, hematopoietic cells, osteocytes, and chondrocytes have similarly been elucidated (Bjornson et al., *Science* 283:534-537, 1999; Tsai and McKay, *J. Neurosci.* 20:3725-3735, 2000; Keirstead et al., *J. Neurosci.* 19:7529-7536, 1999; Mujtaba et al., *Dev. Biol.* 200:1-15, 1998; Clark et al., *Science* 288:1660-1663, 2000).

Our recent discovery that MNSCs maintain the potential to produce both neural and non-neural cell types has been accompanied by the discovery that non-neural stem cells such as bone marrow-derived stem cells (*i.e.*, stromal cells or mesenchymal stem cells) also have the potential to produce a wide variety of neural and non-neural stem cells (Ferrari et al., *Science* 279:1528-1530, 1998; Gussoni et al., *Nature* 401:390-394, 1999; Peterson et al., *Science* 284:1168-1170, 1999; Pereira et al., *Proc. Natl. Acad. Sci. USA* 92:4857-4861, 1995; Prockop, *Science* 276:71-74, 1997; Kessler and Byrne, *Annu. Rev. Physiol.* 61:219-242, 1999; Pittenger et al.,

Science 284:143-147). The conditions under which these bone marrow-derived cells differentiate as, for example, skeletal muscle cells, cardiac muscle cells, hepatocytes, adipocytes, osteocytes, or chondrocytes are likely to be conditions under which the peripheral tissue-derived MNSCs would differentiate similarly. Thus, the peripheral
5 tissue-derived MNSCs described herein can be induced to differentiate into both neural and non-neural cells that are not normally found in the tissue from which the MNSCs were derived.

The foregoing experiments were performed using the following methods.

10 *Skin-derived MNSC culture*

For neonatal (three to 14 days) and adult (two months to one year) mice, skin from abdomen and back was carefully dissected free of other tissue, cut into 2-3 mm³ pieces, washed three times in HBSS, and then digested with 0.1% trypsin for 40 minutes at 37 C, followed by 0.1% DNAase for one minute at room temperature.
15 Tissue pieces were then washed twice with HBSS, once with media (DMEM-F12, 3:1, 1 g/ml fungizone, 1% penicillin/streptomycin) containing 10% rat serum (Harlan Bioproducts), and twice with serum-free media. Skin pieces were then mechanically dissociated in media, and the suspension poured through a 40 M cell strainer (Falcon). Dissociated cells were centrifuged, and resuspended in 10 ml
20 media containing B-27 supplement, 20 ng/ml EGF and 40 ng/ml bFGF (both Collaborative Research). Cells were cultured in 25 cm² tissue culture flasks (Corning) in a 37 C, 5% CO₂ tissue culture incubator.

To culture human skin-derived MNSCs, two to three pieces of scalp tissue ranging between 4-9 mm² (generated by placement of the stereotaxic apparatus for
25 neurosurgery) were washed with HBSS, any subcutaneous tissue was removed, and the skin was cut into small pieces 1-2 mm³ in size. Tissue pieces were transferred to 15 mL Falcon tubes, washed three times with HBSS, and enzymatically digested in 0.1% trypsin for 40 minutes at 37 C, and then washed as for mouse tissue. Dissociated cells were suspended in 5 mL of the same media used for mouse

cultures, with the addition of 20 ng/ml LIF (R&D Systems Inc.). The cell suspension was placed in Falcon 6-well tissue culture plates and maintained in a 37 C, 5% CO₂ tissue culture incubator. Cells were subcultured by partial dissociation of the clusters that formed every 7 to 10 days.

5 To passage floating clusters of cells, the medium containing the cell clusters was centrifuged, the cell pellet mechanically dissociated with a fire-polished Pasteur pipette, and the cells reseeded in fresh media containing B-27 supplement and growth factors as above. Cells were passaged every 6 to 7 days. For induction of differentiation into neural cells, the cell clusters were centrifuged, the growth
10 factor-containing supernatant removed, and the clusters resuspended in fresh media containing B-27 supplement and either 3% rat serum or 1-3% fetal bovine serum. The clusters were then plated onto 4-well Nunclon culture dishes coated with poly-D-lysine/laminin, and the medium was changed every 3 to 7 days.

15 *Transplantation of olfactory epithelium-derived MNSCs*

 Olfactory epithelium-derived MNSCs were purified and cultured as described herein. Female Sprague-Dawley rats or CD1 albino mice (Charles River, Montreal, Quebec, Canada) weighing 180-200 g or 25-30 g, respectively, were anaesthetized with a mixture of ketamine (90 mg/kg) and xylazine (10 mg/kg)
20 (intraperitoneal) prior to stereotactic injections of 24 µg of 6-hydroxydopamine hydrobromide (dissolved in 5 µL of 0.9% saline containing 0.2 mg/ml ascorbate) into the right medial forebrain bundle (Tooth bar:-2.4 mm; A:-4.4 mm; L:1.0 mm; V:7.5 mm). Two weeks after the lesion, animals were tested for rotational behavior. Animals were immunosuppressed with cyclosporine (40 mg/kg, intraperitoneal)
25 once a day until the day of sacrifice. For MNSC transplantation, anaesthetized animals were mounted in a Kopf stereotactic apparatus, and 2 x 2.5 µL aliquots of MNSCs were injected unilaterally into the lesioned caudate putamen or bilaterally in some animals. The injections were made using a 5 µL Hamilton syringe at the following coordinates: Tooth bar, -2.4 mm; A: 0.2; L: 3.0; V: 5.5-6.0. Injections

were performed over a period of three minutes, a further five minutes was allowed for diffusion, and the needle was then retracted. These 5 μ L injections contained MNSCs derived from one neonatal pup cultured for 7 to 14 days. For the BrdU experiments, BrdU (10 μ M) was added to culture media for 18 hours, after which
5 the MNSCs were washed three times with fresh media to remove the BrdU, and then transplanted one day later.

Transplantation of skin-derived MNSCs

Labeling of skin-derived MNSCs was performed as follows. Three days
10 prior to transplantation, free-floating cell clusters were partially dissociated by gentle trituration, and then exposed to 50 MOI of a recombinant adenovirus expressing GFP, using standard techniques. Twenty-four hours later, the MNSCs were centrifuged, washed, and resuspended in fresh medium containing 2 μ M BrdU for an additional two days. Prior to transplantation, MNSCs were rinsed five times
15 with fresh medium and resuspended to a concentration of 50,000 cells/ μ L. At the time of transplantation, approximately 75% of the MNSCs expressed GFP, while 95% were BrdU positive.

MNSCs labeled with BrdU and GFP were stereotactically injected into the right lateral ventricle of cryoanaesthetized two day old rat pups (co-ordinates from
20 Bregma: lateral 1.5 mm, ventral 3.3 mm). Approximately 50,000 cells were injected over a three minute period in a volume of 1 μ L. Fourteen days following transplantation, mice were perfused with 50 mL 4% formaldehyde buffered with PBS. Fifty micron coronal sections through the forebrain were cut using a freezing microtome and analyzed immunocytochemically. All eight animals receiving cell
25 transplants showed extensive labeling for tagged cells. No evidence of tumor formation was observed.

Immunostaining

Immunostaining of olfactory epithelium-derived MNSCs was performed as follows. With the exception of GC immunocytochemistry, culture dishes were washed twice with Tris-buffered saline (TBS; 10mM Tris, 150mM NaCl, pH 8), then fixed with 4% formaldehyde, washed three times with TBS, blocked with TBS plus 2% goat serum (Jackson ImmunoResearch, Mississuagua, Ontario, Canada), and 0.1% Triton-X (Sigma Chemicals, St. Louis MO) for 30 minutes, then incubated with primary antibody in TBS plus 2% goat serum. Following primary antibody incubation, the dishes were washed three times with TBS, incubated in secondary antibody in TBS plus 2% goat serum, washed three times, and then viewed with a fluorescence inverted microscope. The antibodies to GFAP (Boehringer Mannheim, Laval, Quebec, Canada), III tubulin (Sigma), NeuN (Dr. R. Mullen), MAP-2 (clone AP-20; Sigma), and NF-160 (American Tissue Culture Collection, Manassas VA) were monoclonal antibodies used at concentrations of 1:200; 1:25; 1:10, and 1:1 respectively. Antibodies to nestin (a gift from Dr. Ron MacKay (National Institutes of Health), TH (Eugenetech Eugene, OR), and DBH (Eugenetech) were rabbit polyclonal antibodies used at concentrations of 1:1000, 1:200, and 1:200 respectively. Secondary antibodies Cy3 conjugated goat anti-mouse (Jackson ImmunoResearch) and Cy3 conjugated goat anti-rabbit (Jackson ImmunoResearch), and were used at 1:1500. For double-labelling experiments, we used FITC goat anti-mouse (Jackson ImmunoResearch).

For GC immunocytochemistry, living cultures were incubated in DMEM containing HEPES, 5% heat inactivated horse serum (HS), and 1:10 GC antibody for 30 min at 37 C, washed three times with the medium/HEPES/HS, fixed with 4% formaldehyde for 15 minutes, rinsed three times in TBS, incubated in Cy3 conjugated goat anti-mouse antibody (1:1500) for two hours, and finally washed three times in TBS. Cultures processed for immunocytochemistry without primary antibodies revealed no staining.

Immunocytochemical analysis of cultured skin-derived MNSCs was performed as follows. The primary antibodies that were used were: anti-nestin

polyclonal (1:250, Dr. Ron McKay, NINDS), anti-nestin monoclonal (1:400, PharMingen Inc.), anti- β III-tubulin monoclonal (1:500, Tuj1 clone, BabCo), anti-neurofilament-M polyclonal (1:200, Chemicon Intl.), anti-GAD polyclonal (1:800, Chemicon Intl.), anti-NSE polyclonal (1:2000, Polysciences Inc.), anti-GFAP
5 polyclonal (1:200, DAKO), anti-CNPase monoclonal (1:400, Promega), anti-p75NTR polyclonal (1:500, Promega), anti-SMA monoclonal (1:400, Sigma-Aldrich), and anti-A2B5 monoclonal (Dr. Jack Snipes, M.N.I.). The secondary antibodies were Cy3-conjugated goat anti-mouse (1:200), Cy3-conjugated goat anti-rabbit (1:400), FITC-conjugated goat anti-mouse (1:50-1:100), and FITC-conjugated
10 goat anti-rabbit (1:200) (all from Jackson Immunoresearch Laboratories).

Immunocytochemical analysis of free-floating brain sections was performed by DAB immunohistochemistry. For GFP, sections were incubated in 0.3% H₂O₂ for one hour to inhibit endogenous tissue peroxidase activity prior to blocking. For BrdU immunohistochemistry, sections were pre-incubated in 0.5%
15 sodium borohydride for 20 minutes prior to blocking of endogenous peroxidase activity in 0.03% H₂O₂ for 30 minutes. To permeabilize the nuclei for BrdU immunohistochemistry, sections were incubated in 1% DMSO for 10 minutes, the DNA denatured with 2N HCl for 60 minutes, and the HCl neutralized with 0.1M borate buffer for 5 minutes. All sections were blocked for one hour in 10% BSA,
20 and then incubated for 48 hours at 4°C with either anti-GFP (1:1000, Clontech) or anti-BrdU (1:100, Becton-Dickinson). Primary antibodies were detected using a biotinylated horse anti-mouse secondary antibody (1:200, Vector Laboratories) for one hour at room temperature, and visualized using the Vectastain kit (Vector Laboratories) and a nickel-enhanced DAB reaction containing 0.05% DAB, 0.04%
25 nickel chloride, and 0.015% H₂O₂. Sections were mounted onto slides, dehydrated through a series of ethanols and Histoclear (Fisher Scientific), and coverslipped using Permount (Fisher Scientific).

Fluorescence immunohistochemistry was performed to co-localize GFP expression with NSE. Free-floating sections were blocked in 10% BSA for one hour at room temperature, and then incubated 48 hours at 4°C in a solution containing mouse anti-GFP and rabbit anti-NSE. Sections were incubated with Cy3 conjugated anti-mouse and FITC conjugated anti-rabbit secondary antibodies for one hour at room temperature, and coverslipped using Sigma Mounting Medium.

Other Embodiments

The present invention has been described in terms of particular embodiments found or proposed by the present inventors to comprise preferred modes for the practice of the invention. It will be appreciated by those of skill in the art that, in light of the present disclosure, numerous modifications and changes can be made in the particular embodiments exemplified without departing from the intended scope of the invention. All such modifications are intended to be included within the scope of the appended claims.

All publications, patents and patent applications are herein incorporated by reference in their entirety to the same extent as if each individual publication, patent or patent application was specifically and individually indicated to be incorporated by reference in its entirety.

Claims

1. A multipotent stem cell substantially purified from skin of a mammal.
2. The multipotent stem cell of claim 1, wherein said skin comprises the
5 dermal layer.
3. The multipotent stem cell of claim 1, wherein said skin is from a postnatal mammal.
- 10 4. The multipotent stem cell of claim 1, wherein said skin is from an adult mammal.
5. The multipotent stem cell of claim 1, wherein said skin is from a juvenile mammal.
15
6. The multipotent stem cell of claim 1, wherein said mammal is a human.
7. The multipotent stem cell of claim 1, wherein said cell expresses
20 nestin.
8. The multipotent stem cell of claim 1, wherein said cell expresses fibronectin.
- 25 9. The multipotent stem cell of claim 1, which under appropriate conditions can be differentiated into a neuron, an astrocyte, a Schwann cell, or an oligodendrocyte.

10. The multipotent stem cell of claim 1, which can be differentiated into a non-neural cell.

11. The multipotent stem cell of claim 9, wherein said non-neural cell is a
5 smooth muscle cell or an adipocyte.

12. The multipotent stem cell of claim 1, wherein said cell contains a heterologous gene in an expressible genetic construction.

10 13. The multipotent stem cell of claim 11, wherein said gene encodes a therapeutic protein.

14. The multipotent stem cell of claim 11, wherein said gene encodes a cell fate-determining protein.

15

15. A population of at least ten cells, wherein at least 30% of the cells are multipotent stem cells substantially purified from skin of a mammal.

20

16. The population of cells of claim 15, wherein said skin is from a human.

25

17. A composition comprising (i) a multipotent stem cell substantially purified from skin of a mammal, and (ii) a pharmaceutically acceptable carrier, auxiliary or excipient.

18. A cell aggregate comprising at least ten multipotent stem cells derived from skin of a mammal, said multipotent stem cells capable of differentiating as dopaminergic neurons.

19. The aggregate of claim 17, wherein said cells grow detached from the culture substrate.

20. The aggregate of claim 18, wherein said aggregate comprises at least
5 one hundred multipotent neural stem cells derived from skin of a mammal.

21. A method of producing a population of at least ten cells, wherein at least 30% of the cells are multipotent stem cells substantially purified from skin of a postnatal mammal, or progeny of said multipotent stem cells, said method
10 comprising:

(a) providing said skin from said mammal;

(b) culturing said skin under conditions in which multipotent stem cells proliferate and in which at least 25% of the cells that are not multipotent stem cells die or attach to the culture substrate; and

15 (c) continuing culture step (b) until at least 30% of the cells are multipotent stem cells or progeny of said multipotent stem cells.

22. A method of producing a population of at least ten cells, wherein at least 30% of the cells are multipotent stem cells substantially purified from skin of a postnatal mammal or progeny of said multipotent stem cells, said method
20 comprising:

(a) providing said skin from said mammal;

(b) culturing said skin under conditions in which multipotent stem cells proliferate and in which at least 25% of the cells that are not multipotent stem cells
25 die or attach to the culture substrate;

(c) separating said multipotent stem cells from said cells that attach to said culture substrate; and

(d) repeating steps (b) and (c) until at least 30% of the cells are multipotent stem cells or progeny of said multipotent stem cells.

23. The method of claim 22, wherein said population is at least one hundred cells.

5 24. A multipotent stem cell in the central nervous system of a mammal, said multipotent stem cell produced by a method of comprising transplanting a multipotent stem cell substantially purified from the skin of a mammal into the central nervous system of said mammal.

10 25. The multipotent stem cell of claim 24, wherein said mammal from which said cell is substantially purified is said mammal into which said cell is transplanted.

15 26.. The multipotent stem cell of claim 25, wherein said mammal from which said cell is substantially purified is immunologically similar to said mammal into which said cell is transplanted.

20 27. A kit comprising a multipotent stem cell substantially purified from skin of a mammal.

28. The kit of claim 27, said kit comprising a population of cells, wherein at least 30% of said cells are multipotent stem cells substantially purified from said skin.

25 29. A kit for the substantial purification of multipotent stem cells from skin of a mammal.

30. A method of treating a patient having a disease characterized by failure of a cell type, said method comprising administering to said patient a

multipotent stem cell derived from a peripheral tissue, said multipotent stem cell capable of producing neurons and glia.

31. The method of claim 30, wherein said peripheral tissue is skin.

5

32. A kit for use in performing the method of claim 30.

33. A method for treating a patient having a disease characterized by failure of a cell type, said method comprising administering to said patient a differentiated cell that is the progeny of a multipotent stem cell derived from skin.

10

34. The method of claim 33, wherein said skin is from a postnatal mammal.

35. A method for making a neuron that expresses dopamine, said method comprising:

15

- (a) providing a multipotent stem cell derived from skin; and
- (b) culturing said cell under conditions whereby said cell differentiates into a neuron that expresses dopamine.

20

36. A method for making a cell other than a skin cell, said method comprising culturing a multipotent stem cell substantially purified from peripheral tissue of a mammal under conditions in which said cell differentiates as a cell other than a skin cell.

25

37. The method of claim 36, wherein said cell is a neuron, a glial cell, a cardiac cell, a pancreatic islet cell, an adipocyte or a smooth muscle cell.

38. The method of claim 36, wherein said peripheral tissue is skin.

39. A substantially pure composition of multipotent stem cells, which multipotent stem cell differentiates into neural cell types and non-neural cell types.

5 40. The stem cells of claim 39, wherein said non-neural cell type is an adipocyte, a smooth muscle cell or a cardiac cell.

 41. An ex vivo cellular composition wherein at least 30% of the cells are progeny of the multipotent stem cells of claim 39.

10 42. A cellular composition of claim 41, wherein said progeny is undifferentiated progeny.

 43. A cellular composition of claim 42, wherein said progeny is capable of differentiating into a neural or non-neural cell.

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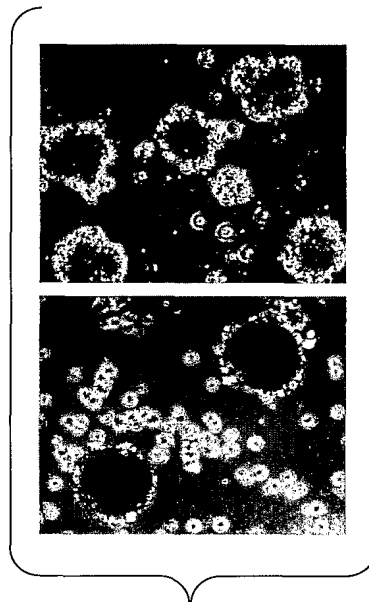


Fig. 1A

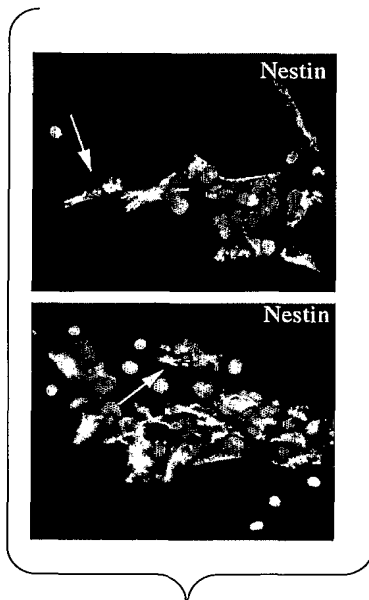


Fig. 1B

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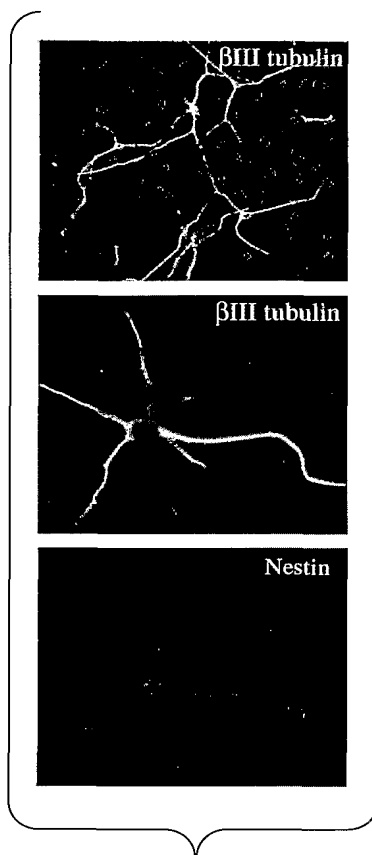


Fig. 1C

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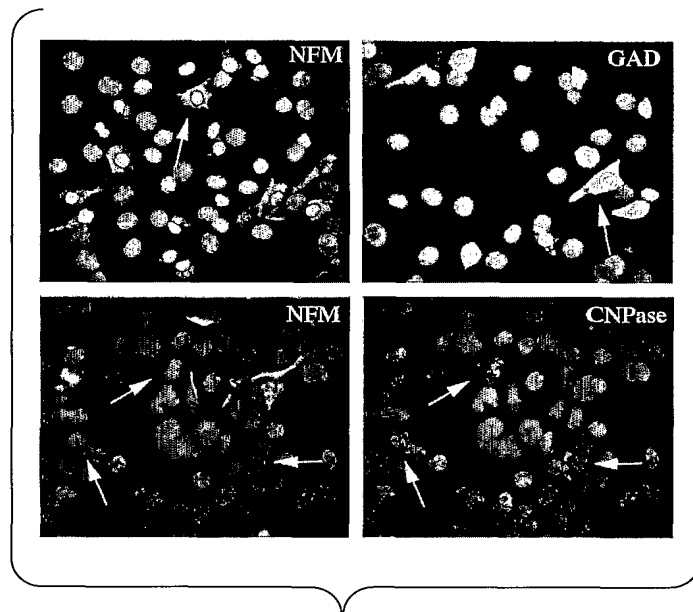


Fig. 1D

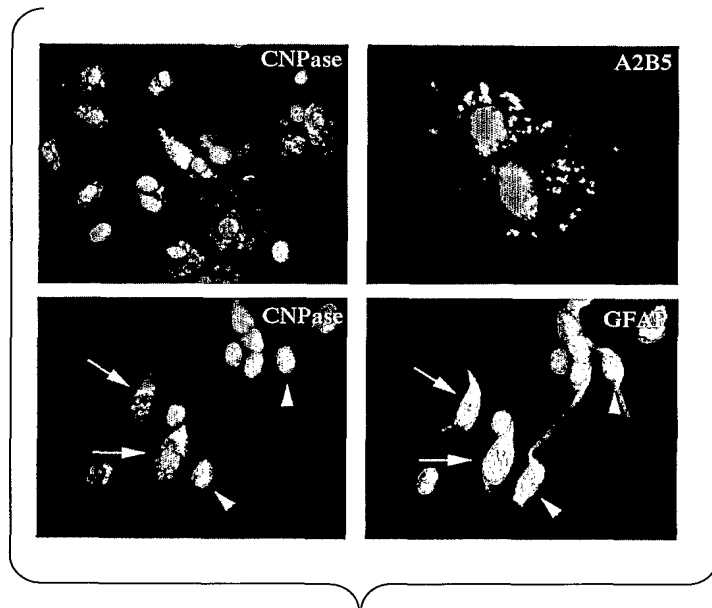


Fig. 1E

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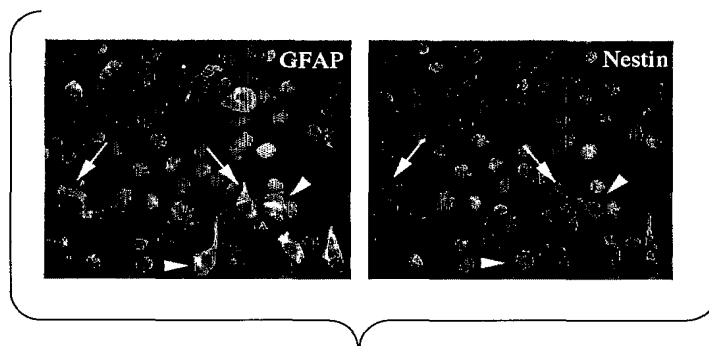


Fig. 1F

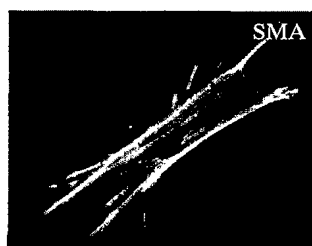


Fig. 1G

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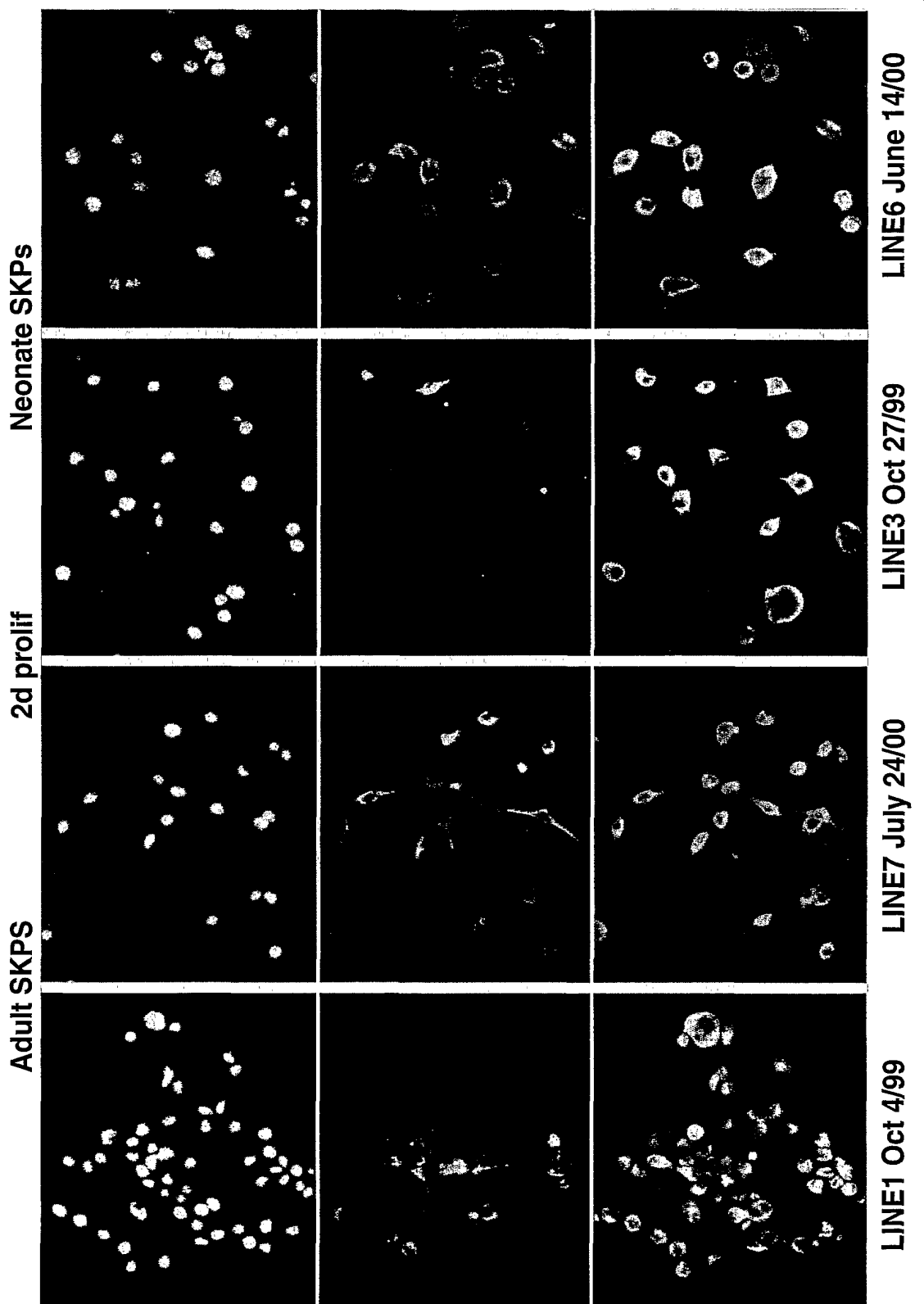


Fig. 2

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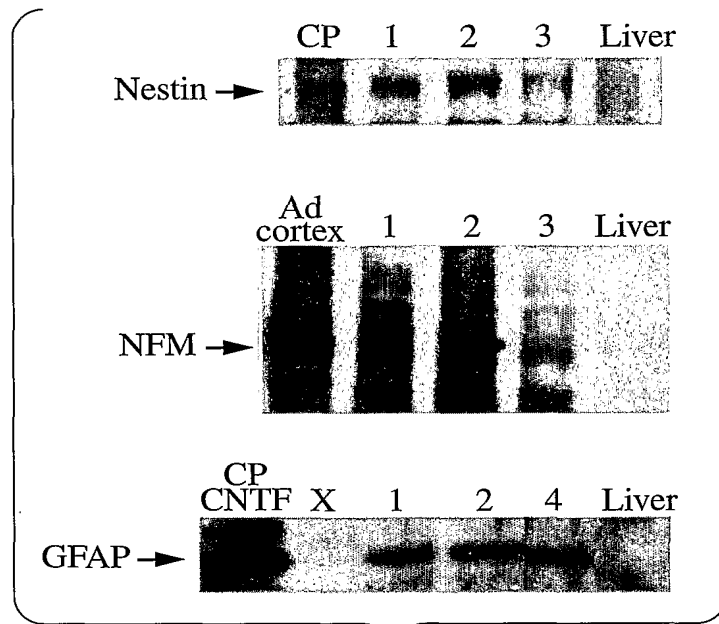


Fig. 3A

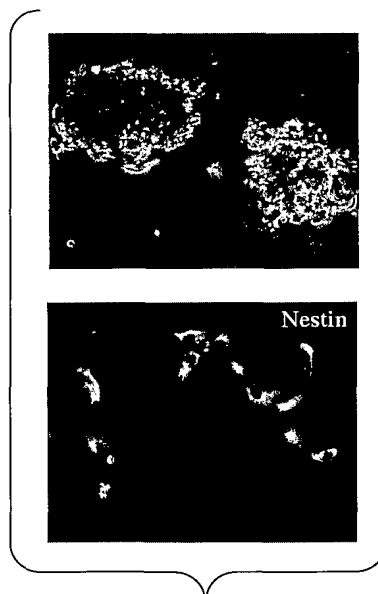


Fig. 3B

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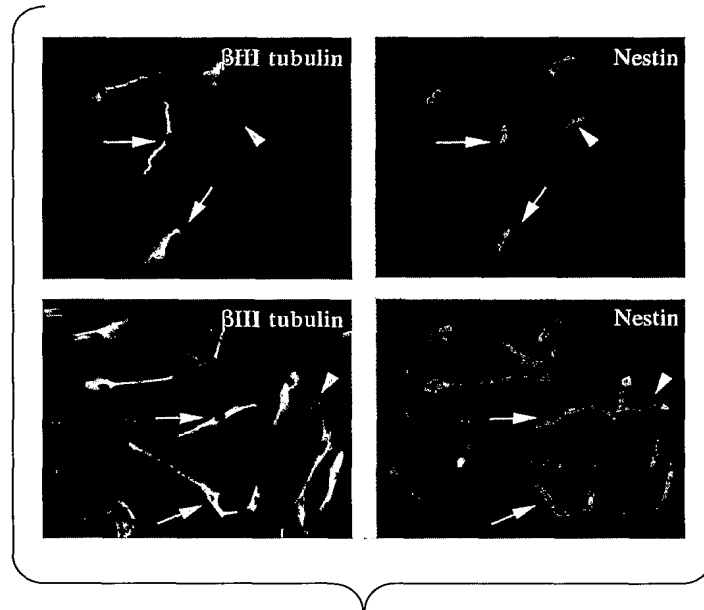


Fig. 3C

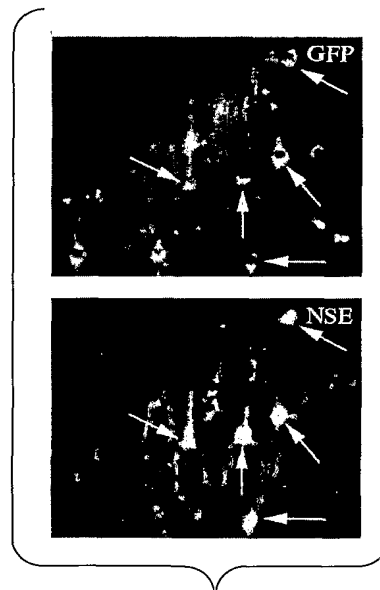


Fig. 3D

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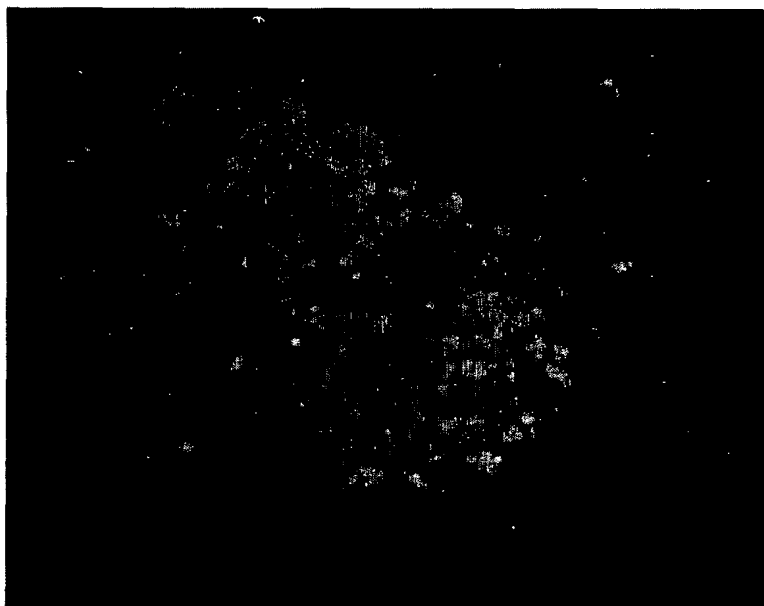


Fig. 4A

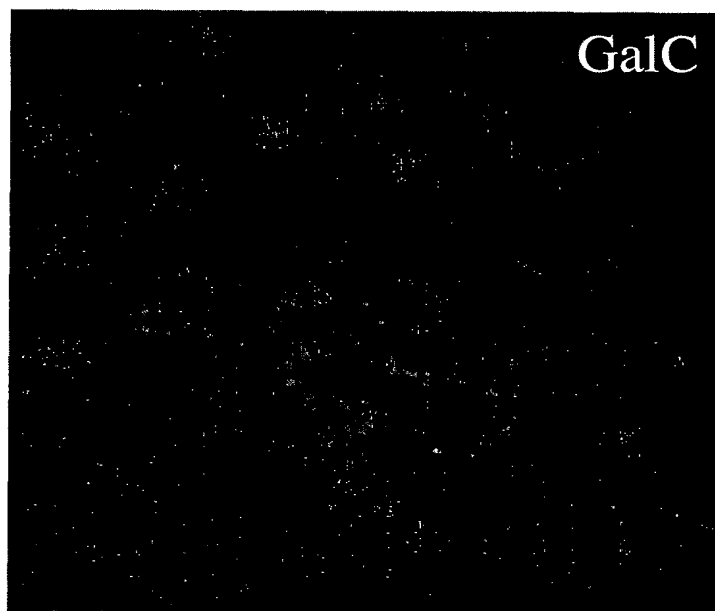
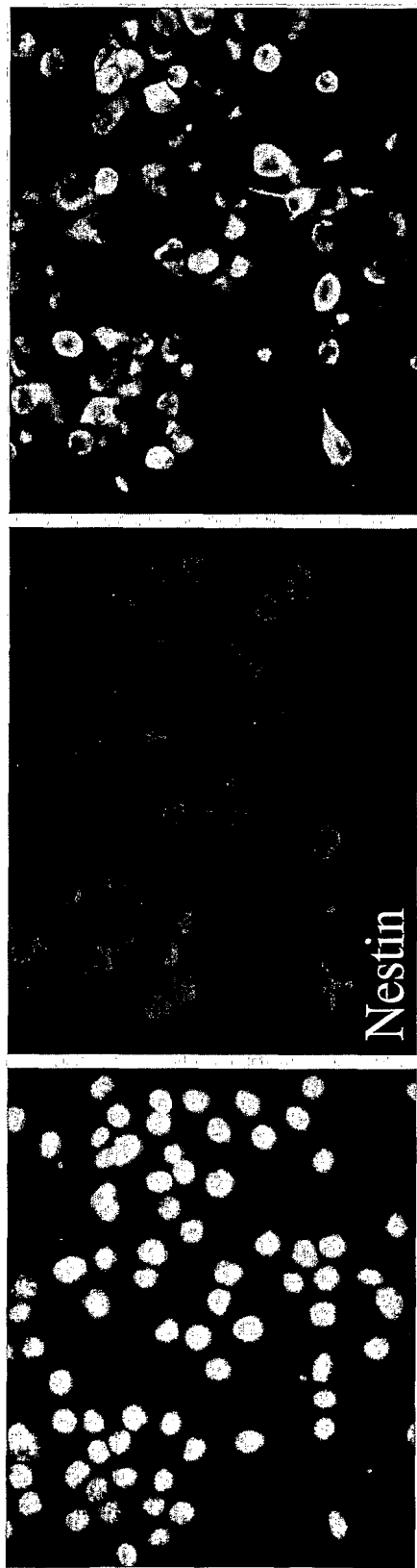


Fig. 4B

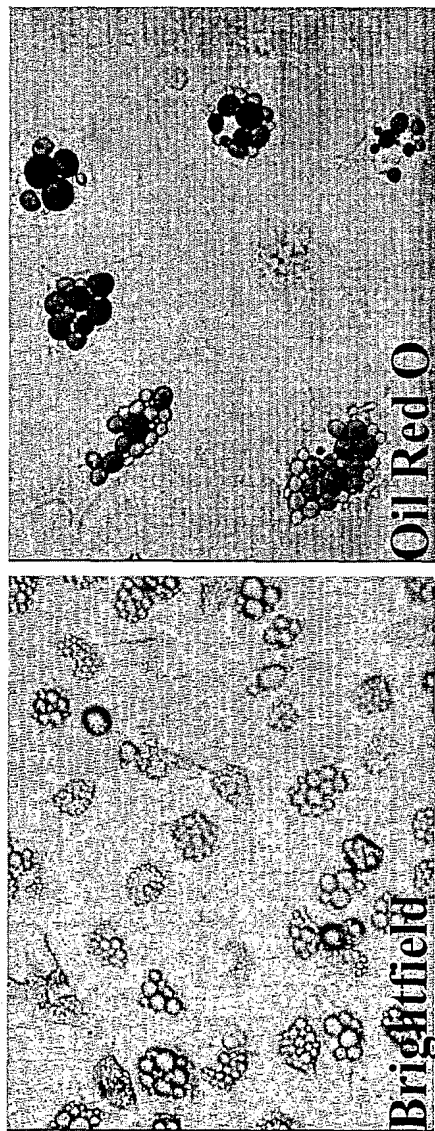
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lating condition: FGF-2 + EGF



Nestin

lating condition: Fetal Bovine Serum



Brightfield

Oil Red O

Fig. 5

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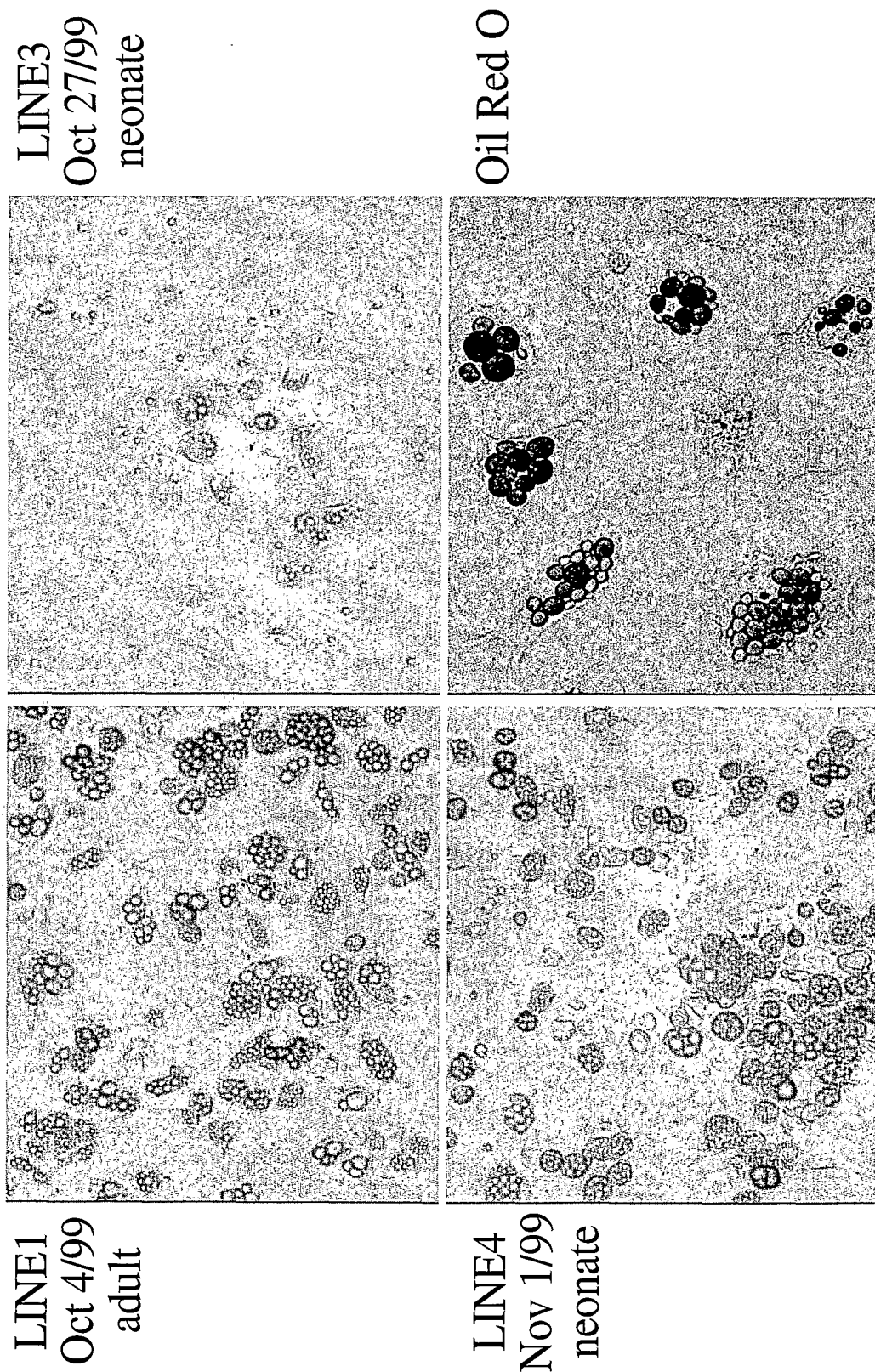


Fig. 6

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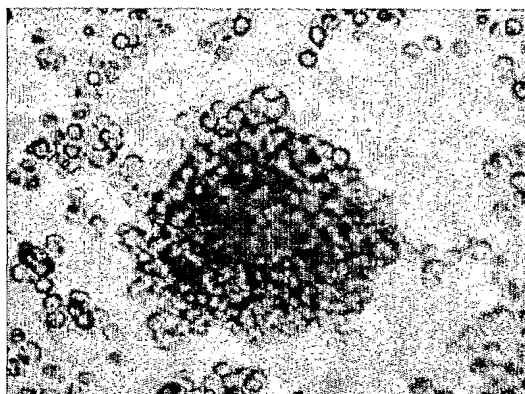


Fig. 7A

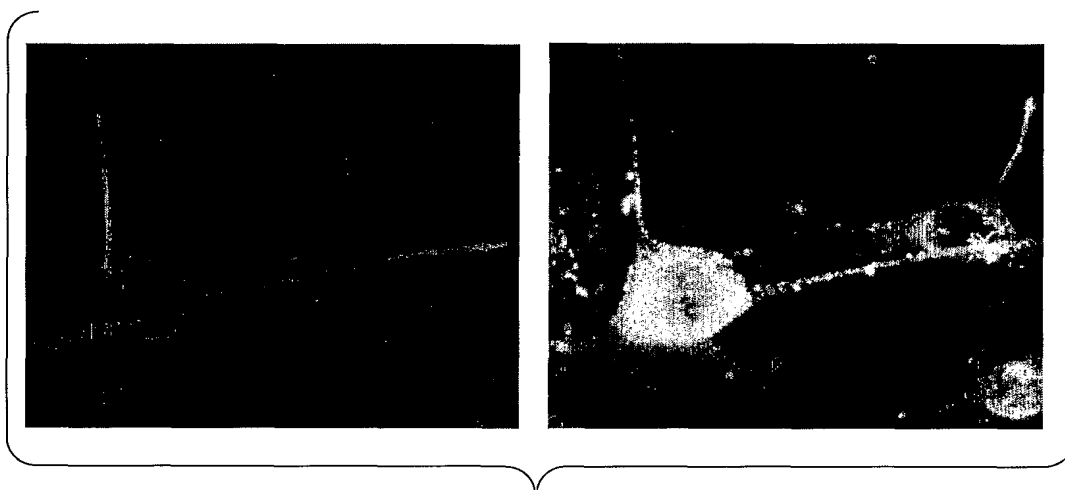
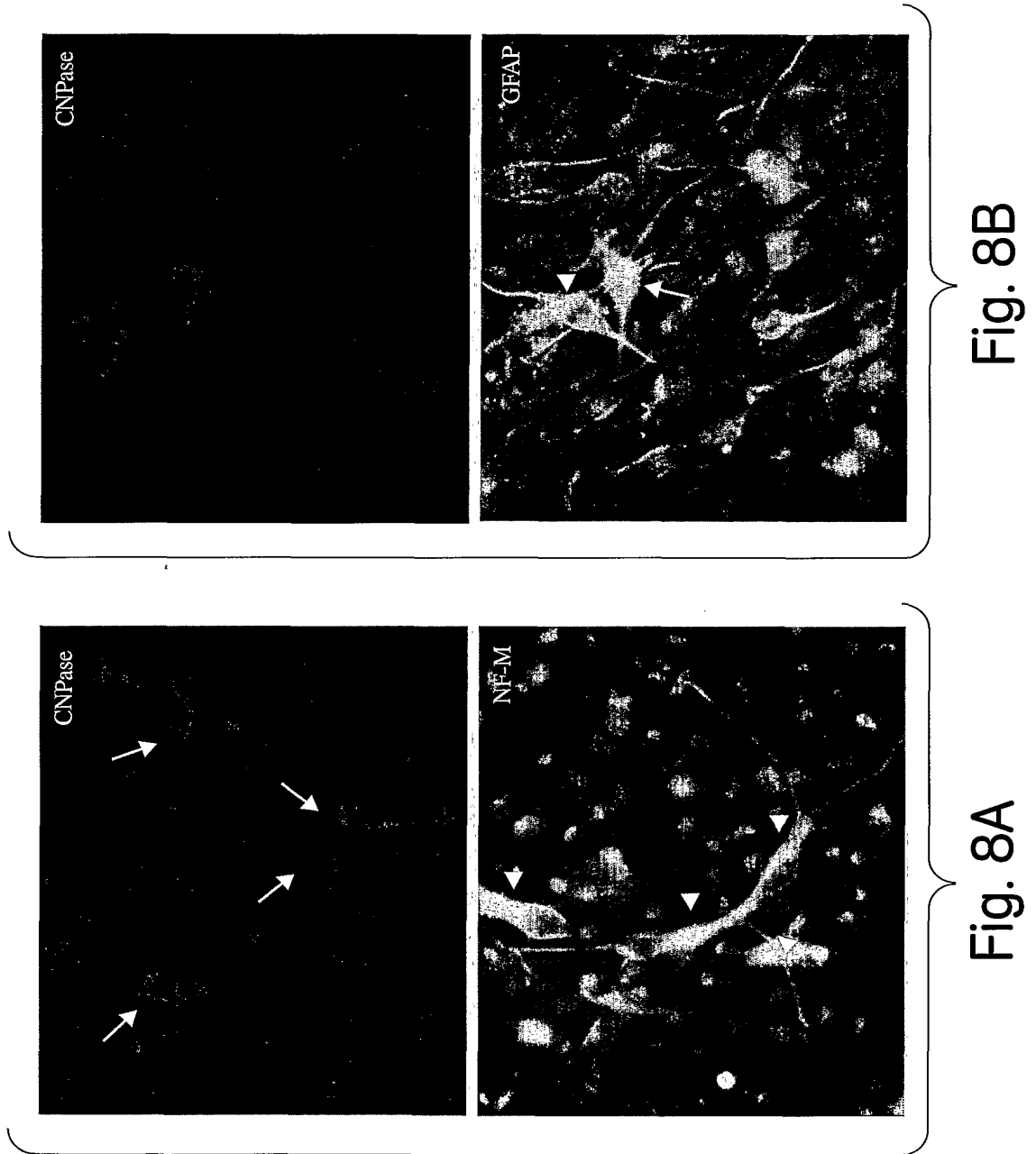


Fig. 7B

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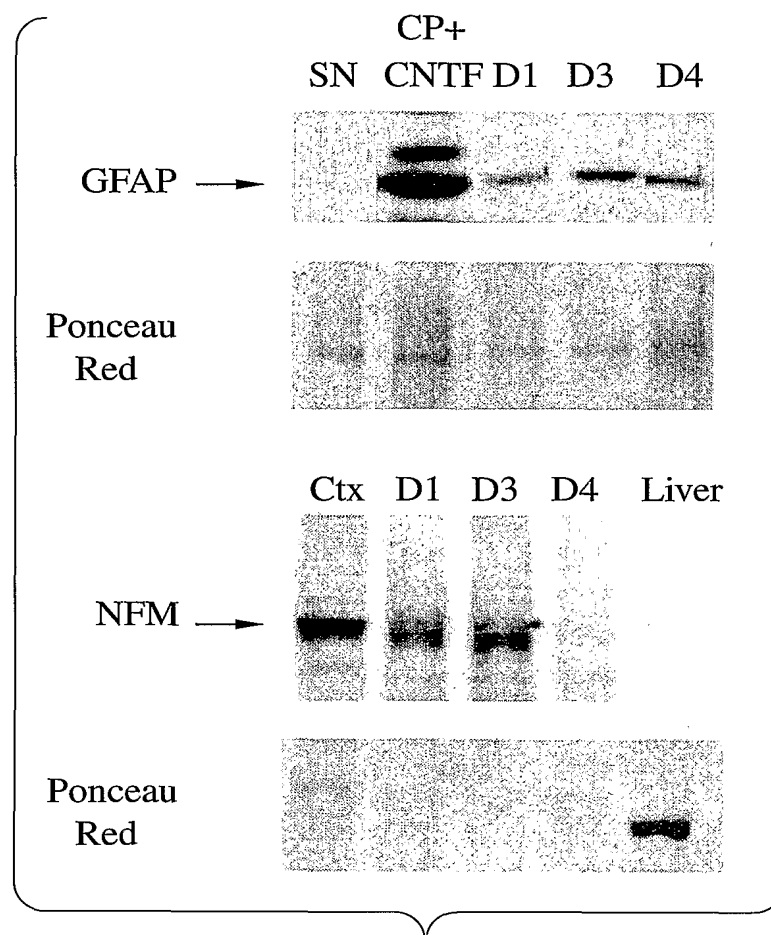


Fig. 9A

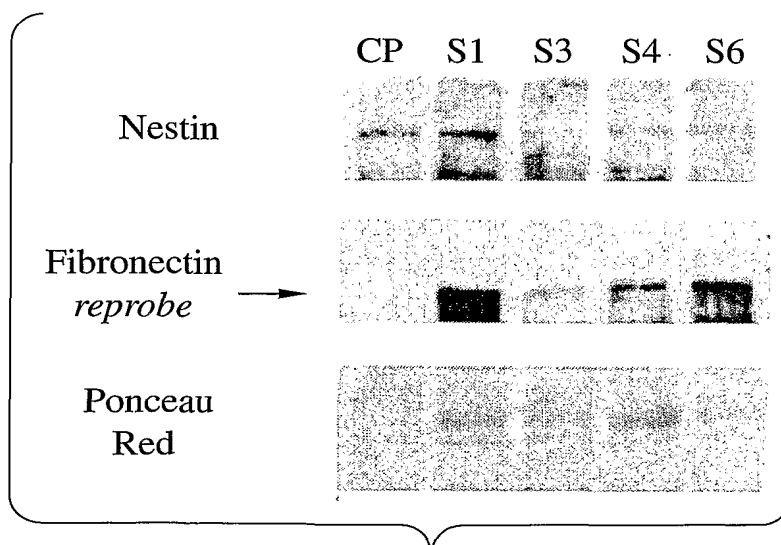


Fig. 9B

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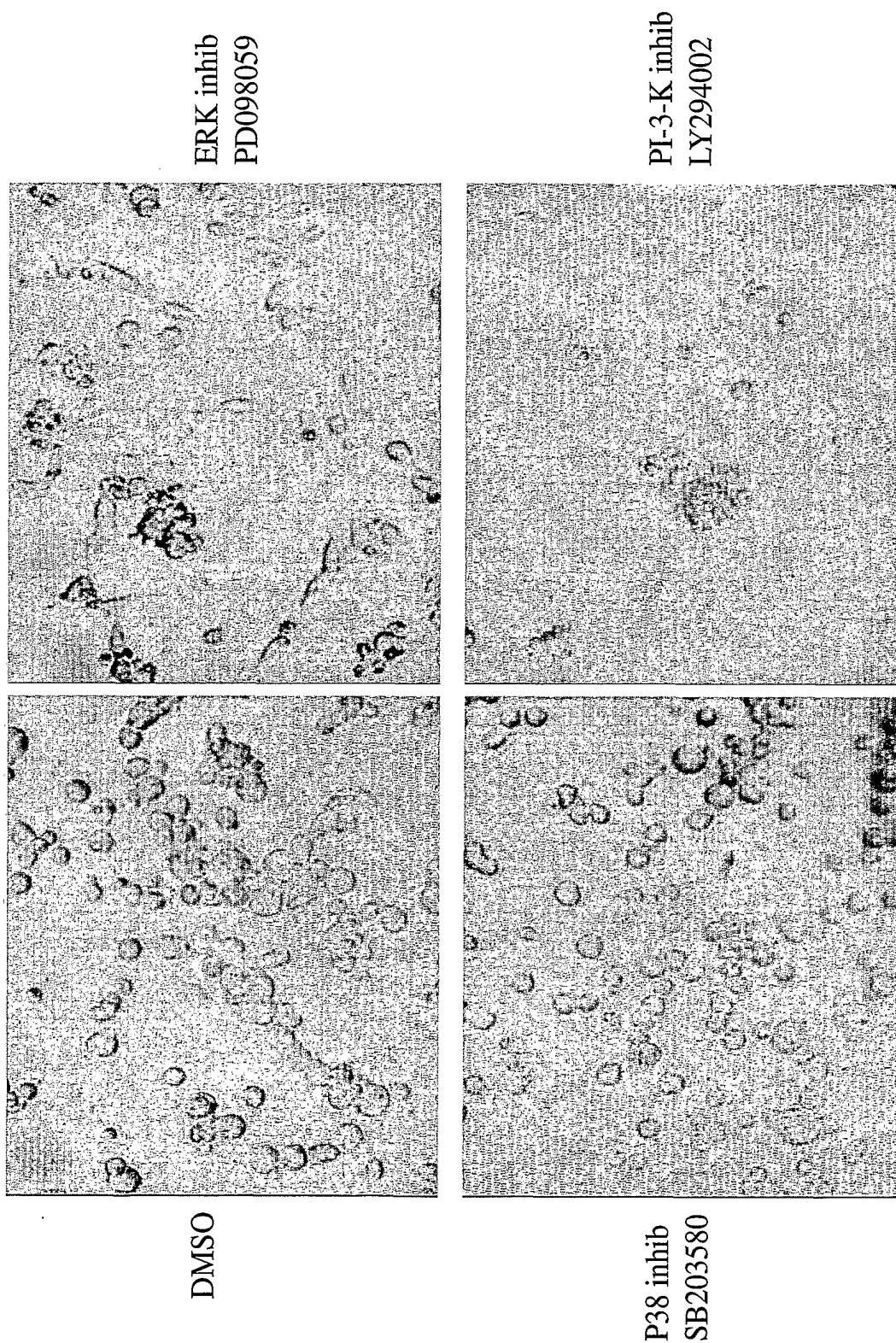


Fig. 10

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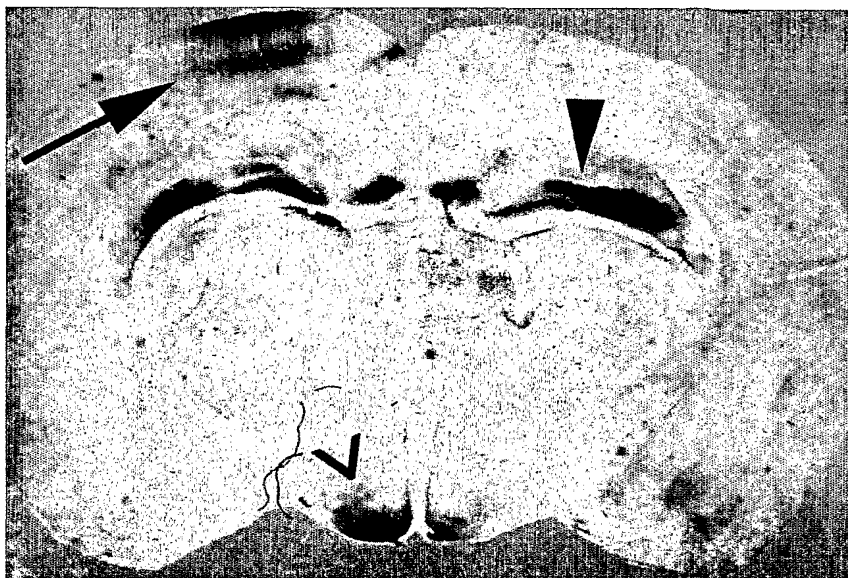


Fig. 11A

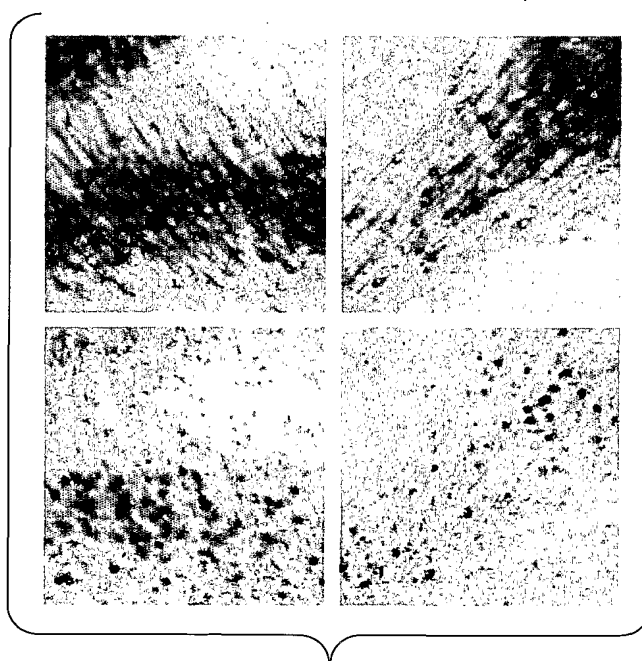


Fig. 11B

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Fig. 11C

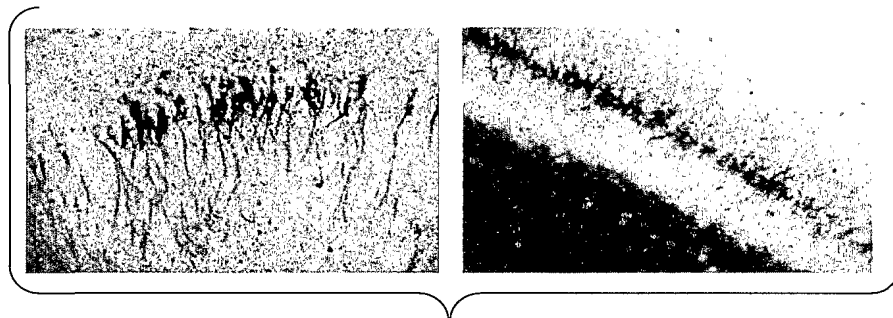


Fig. 11D

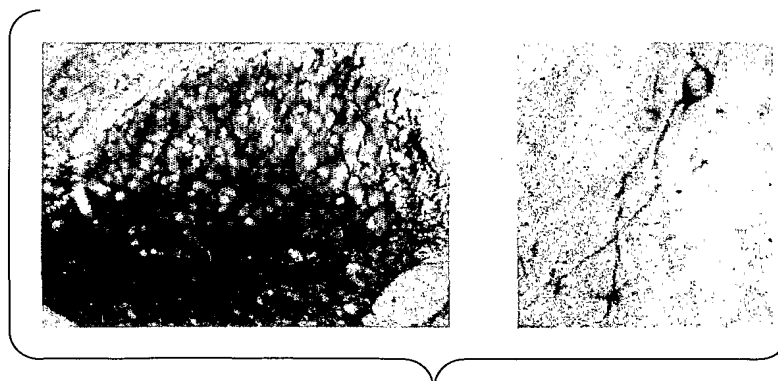


Fig. 11E